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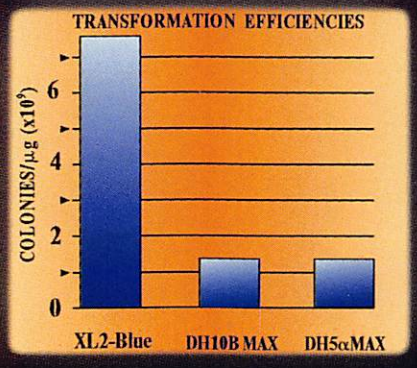

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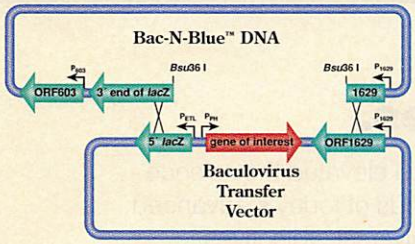

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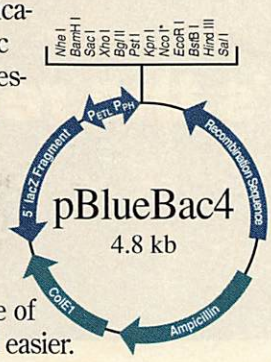

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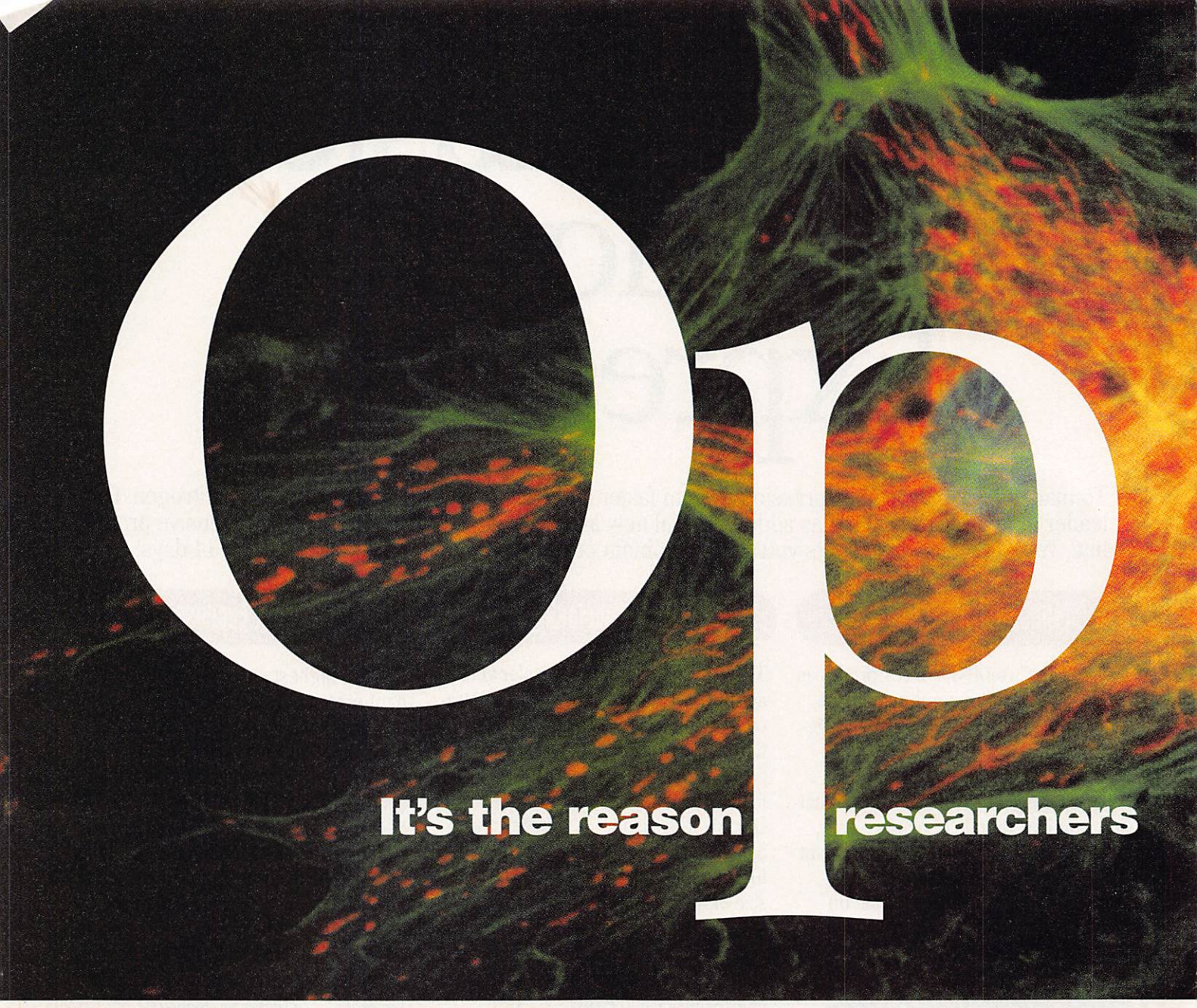

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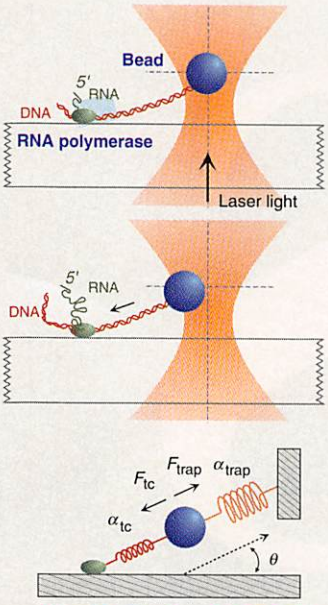

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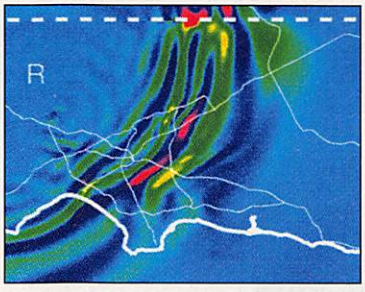

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COVER

MSL-2 is a male-specific protein in *Drosophila* that binds to hundreds of sites along the single male X chromosome and increases gene expression to match that of the two X chromosomes in females. When this protein is ectopically expressed in females, as shown in this immunofluorescence micrograph, the two X chromosomes (paired over most of their length) are decorated in a male-like pattern. See page 1607. [Image: R. L. Kelley and M. I. Kuroda]

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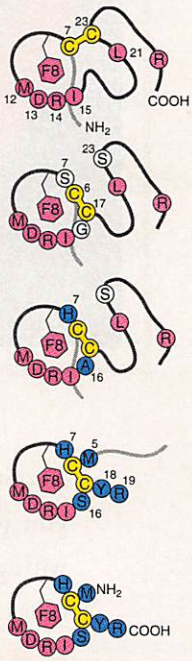

1657
Shrinking a hormone

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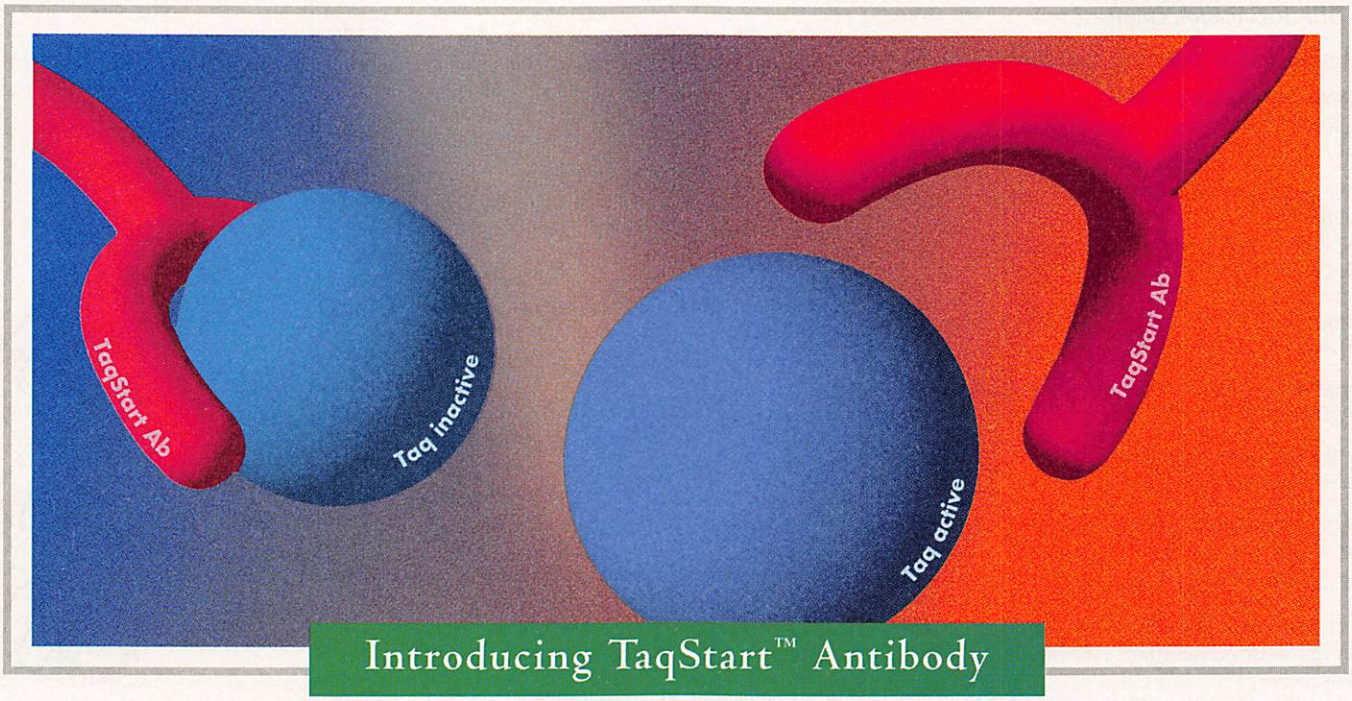

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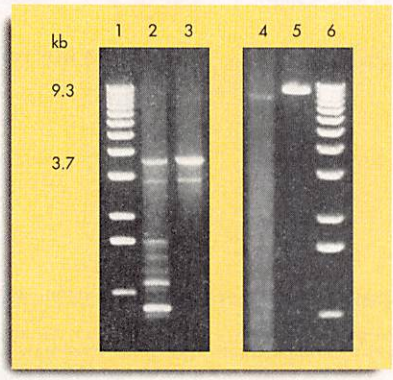

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edited by PHIL SZUROMI

Earthquake simulation

Recent earthquakes in California and Mexico show that major urban areas can experience significant damage and shaking from distant, moderately sized seismic events. Olsen *et al.* (p. 1628) simulated 2 minutes of long-period ground motion after a large earthquake on the San Andreas fault in southern California. Basin shapes and sediment loads in the San Fernando Valley and Los Angeles basins can amplify and prolong shaking compared to other locations at comparable distances from the fault.

Fluctuating flux

The difference between the measured flux of solar neutrinos and that expected from models constitutes the solar neutrino problem. McNutt (p. 1635) reports a correlation between variations in neutrino flux and changes in the solar wind that may indicate a need to revise standard neutrino physics. The correlation is consistent with resonant conversion of electron neutrinos to a nondetected type by interactions with the sun's magnetic field in the corona.

Quantum computers

A computer with logic elements constructed from quantum mechanical systems could in principle factor large numbers into primes, a problem beyond the capacity of conventional computers. However, the algorithm to do this assumes that the quantum computer would be completely isolated. Chuang *et al.* (p. 1633) show that even in the presence of interactions between the quantum computer and its environment that lead to decoherence, such computers may be still realizable. Quantum error correction would be needed to stabilize the computations.

Aerosol source

The stratosphere contains a fine mist of highly concentrated liquid sulfuric acid particles that participate in reactions that can ultimately lead to ozone depletion. Volcanic eruptions inject matter into the stratosphere that can nucleate these particles, but where do the nuclei come from when volcanoes are inactive? Brock *et al.* (p. 1650) show that sulfuric acid–water particles are homogeneously nucleated in the upper troposphere over the tropics and are then transported up into the stratosphere.

Finely fabricated

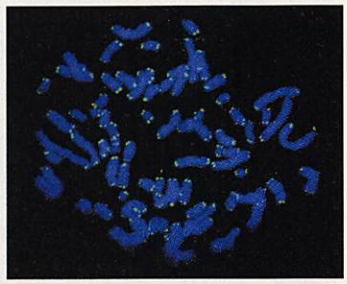
Two reports focus on fabricating micro- and nanoscale devices. Snow and Campbell (p. 1639; see the Perspective by Dagata, p. 1625) can write metal lines and create metal-oxide-metal junctions with an atomic force microscope tip. The conducting tip can reduce TiO_2 to form Ti structures with 10-nanometer features. Lehmann and Stuke (p. 1644) have created three-dimensional microstructures of aluminum and aluminum oxide. A computer directs the movement of a pair of laser beams, and deposition from gas-phase reactants occurs where the lasers cross. They made a linear micromotor that is moved by thermal expansion and contraction; lasers are used to supply the heat.

Hold the salt

The ability of plants to grow in saline soils is correlated with several physiological features, including ion transport. Rubio *et al.* (p. 1660) have identified an aspect of ion transport that is required for normal physiology of the plant but leads to sodium-induced toxicity when the plant is grown in the presence of excessive sodium. The HKT1 ion symporter normally transports K^+ along with Na^+. At high Na^+ concentrations, however, HKT1 mediates additional Na^+ transport through a low-affinity site at the expense of K^+ transport. Mutations in a transmembrane domain resulted in improved ability to discriminate between Na^+ and K^+ and may contribute to improved salt tolerance.

Telomere protein

Chromosome ends, or telomeres, are complexes of double-stranded DNA hexamer repeats and proteins that need to be maintained to avoid DNA damage that can lead to cell cycle



disruption or malignancy. Chong *et al.* (p. 1663) report the cloning of the human telomeric repeat binding factor (TRF). Immunofluorescent labeling showed that the cloned protein localized in vivo to chromosome ends.

Editing and epilepsy

Neurons that express AMPA-sensitive glutamate receptors undergo an RNA editing mechanism whose physiological significance has been unclear. The edited subunits have a lower Ca permeability because of an arginine for glutamine substitution in the pore-forming region of the receptor channel. Brusa *et al.* (p. 1677) produced transgenic mice that could not edit versions of some of their AMPA receptor subunit RNAs. The animals died within 21 days of birth after suffering several seizures. The phenotype of the animals show the importance of RNA editing in the intact mammalian brain and provide a model for early onset epilepsy.

DNA replication origins in higher eukaryotes

In yeast, a multisubunit protein, the origin recognition complex (ORC), binds to replicator sequences and initiates DNA replication. Along with other proteins, ORC determines the frequency of initiation and is also involved in transcriptional silencing. Three reports describe the isolation of ORC-related proteins from higher eukaryotes (see the related news story by Marx, p. 1585). Gavin *et al.* (p. 1667) isolated proteins related to the largest ORC subunits, Orc1p and Orc2p, from multiple organisms and show that the human ORC proteins interact in vivo to form a complex. Gossen *et al.* (p. 1674) cloned the *Drosophila* homologs of Orc2p and Orc5p and find that DmOrc2 is most abundant during embryogenesis, the period of highest DNA replication. Ehrenhofer-Murray *et al.* (p. 1671) show that DmOrc2 can complement a silencing defect in the yeast *orc2-1* mutation but does not complement the replication defect, showing that these functions are separable.

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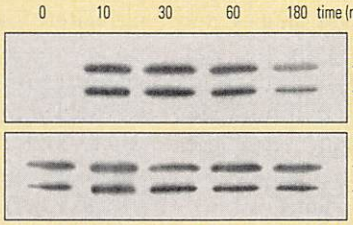
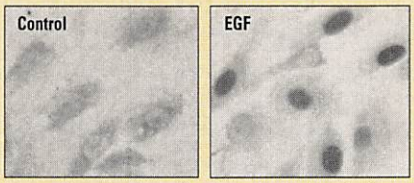


Western Blotting: Quick and easy detection of MAP kinase activation (on Tyr) from SDS-lysates of total PC12 cell extracts using **A.** phospho- and **B.** control-MAP kinase antibodies.

NIH 3T3 CELLS/PHOSPHO-MAPK Ab



Immunohistochemistry: Phospho-antibody allows *in situ*-detection and subcellular resolution of Epidermal Growth Factor (EGF)-induced MAP kinase activation and nuclear translocation.

And cdc2 Kinase

Western Blotting of cell extracts from Soac cells treated with Hydroxyurea (G1/S) or Nocodizate (G2/M) with **A.** phospho- cdc2 (Tyr15) **B.** control cdc2 antibody.

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EDITORIAL

Homage to the Chromosome

Biological organisms, unlike complex inanimate systems, contain information that regulates their day-to-day activities and, more remarkably, lets them produce new organisms from single cells. That this information resides in the chromosomes was established over 75 years ago when it was shown that the cytologically visible chromosomes were distributed at cell division, meiosis, and fertilization in the same patterns as were the genes detected by breeding experiments. Missing from the classical account, however, was any understanding of the nature of the information carried by the chromosomes. That gap was, of course, filled by later spectacular advances in molecular genetics, which showed that a gene is a segment of DNA whose linear sequence of nucleotides specifies the linear sequence of amino acids in a protein. Given the triplet code that predicts amino acids from nucleotides, anyone can read the book of instructions contained in the chromosomes. But just as a book is more than a random assortment of words, chromosomes are more than simple repositories of gene sequences. They must contain regulatory information for turning genes on and off and they must control their own replication, repair, and packaging, as well as the complex movements they carry out during mitosis and meiosis.

Today's issue of *Science* contains five review Articles and four Reports that focus on some of these global properties of chromosomes that cannot be predicted from a knowledge of gene structure and coding alone. Central to any genetic system is the mechanism for moving the chromosomes from place to place. In higher organisms, this involves interaction between the mitotic spindle and a specific part of the chromosome, the centromere. Pluta *et al.* (page 1591) summarize recent information on the (noncoding) DNA sequences at the centromere as well as the special proteins that bind to this part of the chromosome. Although centromeres have often been thought of as passive structures, McKim and Hawley (page 1595) review new evidence that suggests that centromeres take an active role in establishing the spindle and in controlling the molecular "motors" that move the chromosomes to the poles.

Zakian (page 1601) reviews recent studies on telomeres, the specialized ends of chromosomes. Most chromosomes have a repeated 6–base pair sequence at their ends, which is replicated by an enzyme known as telomerase. Telomerase is remarkable in that it is a ribonucleoprotein whose RNA moiety contains the template for the 6-base repeat. Like centromeres, telomeres contain specific proteins not found elsewhere on the chromosome. In a related Report, Chong *et al.* (page 1663) describe a human telomere-binding protein.

The review by Kelley and Kuroda (page 1607) deals with dosage compensation—the raising or lowering of overall gene activity on the X chromosome to compensate for the different number of X's in females versus males. In the fly *Drosophila*, the single X of the male works harder, whereas in the nematode worm *Caenorhabditis* the two X's in the female slow down. In both cases, special proteins coat the affected X chromosomes. In mammals, dosage compensation is accomplished in still another way, by overall inactivation of one of the two X chromosomes in each cell of the female. In her review, Barlow (page 1610) describes a similar phenomenon—gametic imprinting in mammals. In this case, single genes are inactivated instead of a whole chromosome, but only when inherited from a specific parent, sometimes the father, sometimes the mother.

This issue does not contain a review on chromosome replication, but the interested reader can find here three Reports dealing with the important first step in DNA replication, the formation of an initiation complex between DNA and a set of specific proteins [Gavin *et al.* (page 1667), Ehrenhofer-Murray *et al.* (page 1671), and Gossen *et al.* (page 1674)].

What are the common themes of these papers? First, they emphasize the importance and diversity of chromosomal proteins that serve both structural and regulatory roles, most often by interacting with specific but noncoding DNA sequences. Second, they underscore the extraordinary power of genetics to identify the genes that encode these chromosomal proteins. Finally, they provide novel molecular insights into some of the oldest and most baffling problems of chromosome structure and behavior. Chromosome research is alive and well!

Joseph G. Gall

The author is American Cancer Society Professor of Developmental Genetics in the Department of Embryology at the Carnegie Institution of Washington in Baltimore, MD 21210.

Research Notes from
Nikon

Need for Finer, More Detailed Images Puts the Focus on Fluorescence

Until fairly recently, brightfield and phase contrast were the most popular techniques used for microscopic studies. These techniques work well for studies of individual cells, but they are not adequate for imaging smaller entities such as some cellular organelles, proteins, antibodies, microtubules, RNA and DNA. Because fluorescence permits selective imaging and analysis of these substances with resolution down to several nanometers, it is becoming increasingly popular. Recent advances in optics design promise to make it an even more powerful technique.

As biomedical researchers continue to explore smaller and smaller entities, fluorescence microscopy is proving to be an exceptionally powerful tool.

Because fluorescent dyes, or fluorochromes, are absorbed selectively by different substances within a specimen, each substance can be targeted and observed separately. This has made possible techniques such as Fluorescence *in situ* Hybridization (FISH), for example, which has played a key role in identifying chromosomes and determining the number, size and location of specific DNA sequences in mammalian cells.

Smaller objects, finer details

Using fluorescence, scientists can resolve images that cannot be observed with ordinary light. The diameter of a single DNA molecule, for example, is about 2nm, while the finest detail that can be resolved by optical lenses is about 200nm. However, if a fluorescent dye is conjugated with the DNA molecule and the specimen is viewed with a fluorescence microscope, the molecule can be easily detected and measured.

Although it is a very powerful and relatively easy to use tool, fluorescence places extreme demands on the microscope's optics. The near-ultraviolet wavelengths that are used to excite the specimen are very high in energy. At the same time, the light emitted from the specimen is of much lower intensity than the light used to excite it, so the imaging optics must be designed to transmit as much light as possible. Care must also be taken to prevent autofluorescence emissions, which can mask and rob contrast from the image.

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Nikon is the only microscope manufacturer to formulate and manufacture its own glass, and has taken advantage of this capability to create these truly special objectives. They also feature new optical cements and high transmission coatings to permit brighter images and broader wavelength ranges (UV - Deep Red) that are color aberration free with extremely high contrast and low background autofluorescence.

The new CF PLAN FLUOR Objectives also offer higher numerical apertures to capture more of the available light emitted by the specimen. The N.A. of the 40X PLAN FLUOR, for example, is 0.75 compared with 0.7 for the 40X Plan Achromat. The N.A. of the 100X oil PLAN FLUOR is 1.3 compared with 1.25 for the 100X Plan Achromat oil.

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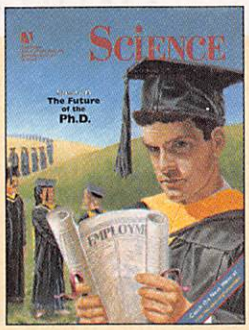

Career issues

The mail was heavy in reaction to the 6 October issue, "Careers '95: The Future of the Ph.D." Several letters expressed the belief that scientists should be in their professions for the sheer love of research, not for a job. Others suggested that those who pursue scientific careers should expect no guarantees about employment. Some proposed alternative ways that science Ph.D.'s could be employed—in business, government, law, or journalism, for example. One correspondent would like to see the position of "postdoc" eliminated altogether, to be replaced by permanent positions that would give a greater sense of security to new Ph.D.'s. The consensus was that a graduate degree in science should qualify one to contribute to society in a variety of useful ways.

Ph.D. Employment

Given the employment situation in science, I believe it would be in the best interests of our field, and of young and mature scientists, if the position of postdoc were eliminated. This could be done by universities, national laboratories, funding agencies, and accreditation agencies working cooperatively or independently. In a field with the economic prospects of ours, to suggest that a new Ph.D. needs additional education before he is really qualified to work is laughable. Naturally, a scientist's education never ends, and there are many ways a scientist could profitably spend time in different universities or national labs. However, such visits are best arranged as exchanges between employed persons.

Many universities have research staff positions, and many national labs contract out work. Universities may be able to use independent contractors also. This way the recent Ph.D. would be hired into what at least may be a permanent position. If he does not work out, he can be terminated, just like any other employee. On the other hand, as grant support shifts, the company or research staff might or might not want to retain the person.

It is probably true that with the available support more postdocs can be employed than permanent employees. However, this could be considered even more of a reason to eliminate the postdoc position. If the field cannot support a scientist, it is better that he should know it after he completes his Ph.D. than after he has bounced around the country for 10 years, completing five postdocs.

Wallace M. Manheimer
Senior Scientist for Fundamental Plasma Processes, Naval Research Laboratory, Code 6707, Department of the Navy, Washington, DC 20375–5000, USA

I would like to propose a somewhat heretical solution to the career trauma facing young scientists: paying postdocs to embark on nonresearch careers. Individuals adventurous enough to leave research science should be assisted and encouraged. Call it a "G.I. Bill for Postdocs."

An appropriate mechanism would be a federally mandated requirement that all bodies funding research allocate 5% (or more) of their salary budget to fund postdocs seeking to leave research through higher degrees such as the M.B.A. (Small concessions such as this would greatly alleviate the sense of guilt that many principal investigators now feel when they train young scientists.)

Under such a system, a young scientist wanting to go into business, computing, government, teaching, or journalism would have the chance to obtain the formal qualifications and the contacts that are usually essential in such a venture. This would afford them the best possible chance in a new profession and would leave them with a rare combination of training that would enhance the range of contacts between science and other professions. This depth of contact would invigorate science and greatly enhance the "science constituency" within influential sectors of the economy, at the same time securing a brighter future for research and easier access to jobs for tomorrow's graduate students.

Michael Burnet
Laboratoire de Biologie de la Rhizosphere, Institut National de la Recherche Agronomique, 78026, Versailles, Cedex, France

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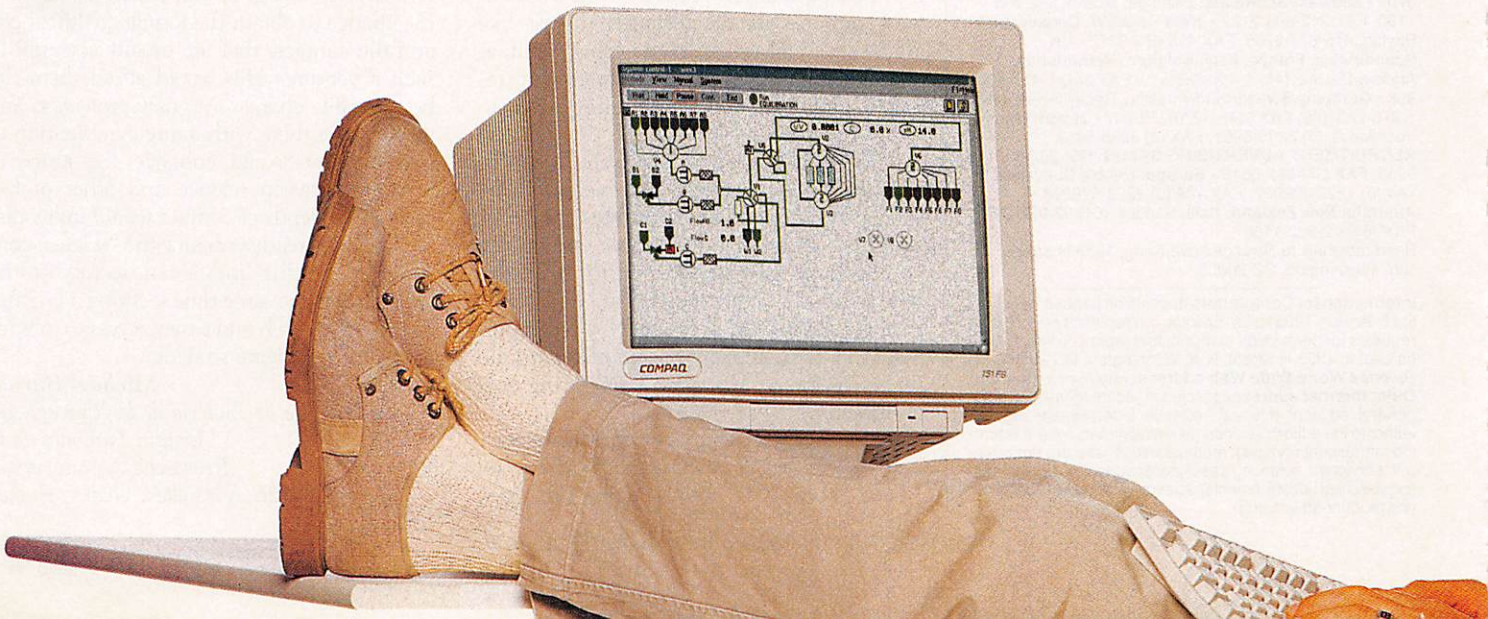

It is reasonable for faculty to express concern that we are training too many Ph.D.'s. Individuals who aspire to obtain a Ph.D. are commonly enthusiastic about research and eager to channel their creative skills into productive work. It is counterproductive to dampen this enthusiasm with clouds of uncertainty about the future of academic research. The discoveries of tomorrow may improve job availability. Moreover, the intelligent, creative, and enthusiastic Ph.D.'s of tomorrow are quite likely to be the people to open new opportunities for research in academics, business, politics, or areas yet to be defined. It would be a significant loss to the progress of society to channel these individuals into less creative career paths.

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Many students embark on the study of a discipline because they love the discipline, and not because they regard it as a sure way to make a living. It is an avocation rather than a vocation. In many sciences, graduate students are needed by professors to accomplish their own research. This raises the spectre of exploitation: Is it just to encourage students to undertake graduate work because we need them in our laboratories, even though there is no prospect of all of them being our successors?

It is certainly unjust to mislead a student as to his prospects for employment. But it is not unjust to offer a student employment as a research assistant as a way of helping the student to follow his avocation.

It has been argued that too much education is a bad thing and that overeducated citizens are a source of unrest and dissatisfaction. It can also be argued that there is no such thing as an overeducated citizen: there are only those who have acquired false expectations in the pursuit of learning rather than true education. For that, I think, we may justly blame ourselves, and resolve to do our jobs better.

Henry E. Kyburg Jr.
Departments of Philosophy and Computer Science, University of Rochester, Rochester, NY 14627, USA

In answer to Don S. Doering's question (Editorial, 18 Aug., p. 903), "How do we fix a system that ... has produced many more Ph.D.'s than the market can bear?", William Lockeretz (Letters, 13 Oct., p. 219) proposes a scheme of rationing to be executed by scientific research professors whose students become the Ph.D. scientists. Although he offers an essentially quantitative goal for the scheme, Lockeretz makes no suggestion as to how the selection from among potential Ph.D.'s is to be effected. About half a century ago there had for at least a decade been in place, as described below, a self-regulating system for producing Ph.D.'s in science that required no selection process and no rationing; and in light of external circumstances, that is, the job market, it was successful. I am convinced that a like system would be successful in our time and beyond.

A single addition to the present mode of producing Ph.D.'s in science should be sufficient to reestablish the old system's essential equivalent: Every student embarking on a scientific education must be made unequivocally aware that there is no guarantee of employment in a field of one's choice as consequence of one's studies—not even with a Ph.D. Let each become imbued with the spirit of the following injunction: "Study this field because you love it. If eventually you are sustained by a career in it, so much the better; if not, the career's failure to materialize must neither surprise nor bruise you. Bear no resentment, but cherish the experience and insight your education will have brought you."

In the late 1930s, physics majors knew of no guarantees of eventual employment in physics. Nor was a particular group of graduate students surprised in 1941 on being informed by its department chair that "this is a physics department, not an employment agency."

Research professors must be—compelled to be, if necessary—persistently frank with their students with regard to career prospects. What has evidently long been a practice of sustaining unjustifiedly sanguine future-career illusions in graduate science departments must be halted. Establishment of a rigorous "no-career-guaranteed" understanding within each graduate science department would likely entail (i) a reduced, if not eliminated, production of many more Ph.D.'s than the market can bear; (ii) a greater proportion of Ph.D. candidates who are in the pursuit for the love of it; and (iii) a lowering of the number of bruised, resentful science Ph.D.'s among those who are obliged to seek employment outside their fields of preference.

Robert Weinstock
Emeritus Professor, Department of Physics, Oberlin College, Oberlin, OH 44074, USA

I share with Doering and many other investigators the panic over future employment opportunities within academia. Not only do

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I worry about the future of my own graduate students and postdocs, I have even more worries about the implications for the country as a whole.

Doering seems to suggest that we train Ph.D.'s for three reasons. The first is to obtain cheap labor to get our science done. The implications here are beyond comment. I would hope that kind of attitude does not exist, and if it does, we should wipe it out. The second justification given for training Ph.D.'s is that we need cheap labor to instruct undergraduates. Again, the implications about the morality of the academic community are disturbing. If this is happening, we should be working to change the structure of undergraduate education. Why do we need so many graduate students to teach so many undergraduates if the market is dictating a need for fewer people trained in science? The third reason, and in my opinion, the most disturbing one of all, is that we train people so that they can take jobs. I am not convinced that this should be a major goal. People can pursue learning for many reasons, only one of which is to secure employment.

Stephen M. Schwartz
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Academic Expressways

I was taken aback by the headline "Scientists enjoy life in the not-so-fast lanes" (Karen Celia Fox, Careers '95, 6 Oct., p. 141). It is true that my colleagues do very much enjoy their lives. It is also true that they are very much in the fast lanes, but on an expressway that is different from those where faculty at research universities are found. Faculty at many undergraduate colleges must run very hard to achieve excellence in both instruction and research. Their product, their students, are the graduate students, business leaders, and educated citizens upon which our nation depends. My institution is often the leading producer of B.A./B.S. chemistry majors in the nation. In the past 10 years 11 graduates have earned one of the 50 National Science Foundation (NSF) graduate fellowships granted annually to graduates of 4-year colleges. All of my colleagues hold or have recently held peer-reviewed research grants, and most have substantial publication records. They are also excellent teachers and some are national leaders in curriculum reform. Nearly all have been awarded grants by NSF for instructional equipment or curricular development. Three members of our chemistry faculty have been awarded the Catalyst Award of the Chemical Manufacturer's Association. As the article indicates, faculty at undergraduate institutions have different goals, and they have chosen a different route; there is substantial evidence that their work in the fast lanes contributes in an important way to the nation.

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■

What Is Excellence?

The Careers '95 issue raises the spectre of the "versatile Ph.D." Definitely, broad experience enables a committed and restlessly curious graduate student to attack a lifelong research question from many angles. But I suspect that what is meant is conferring on those graduate students who do not have a burning curiosity the ability to provide skilled hands for any of a variety of employers. If our programs are versatile, we will still need a focused program for students who have always known they wanted to uncover mysteries. We already

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have such a program: it is called the Ph.D. Versatile expertise is the provenance of the M.S.

It is perhaps not accidental that the view that excellence does not require focused self-determination arises simultaneously with the view that excellent research can be predicted and scheduled, that an effort of 5% on the part of a principal investigator on a project suffices, and that the prolonged adolescence of apprenticeship should continue into the postdoc and perhaps "captive assistant professorship." For committed graduate students wondering whether it was always this way, the answer is "no." For student or professor looking for a little insight into the requirements for excellence, I recommend Gertrude Stein's essay "What are masterpieces and why are there so few of them?"

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■

Resubmission of NIH Grant Proposals

I read with dismay the latest proposal by the National Institutes of Health to streamline the grant process by disallowing more than one resubmission of a grant (ScienceScope, 6 Oct., p. 19). This is ironic since it is admitted that this policy would have a small impact on reducing paperwork. As a current member of several granting agency study sections, I have observed that the review committees frequently do not fund a potentially interesting and important grant submission in part because an element has not been fully expanded or proposed. This points to the larger reason as to why grants are being repetitively submitted—there are insufficient resources to fund many fine grant proposals. In fact, there is often nothing intrinsically wrong with a proposal; rather it is the policy of support for research in this country that "limits enthusiasm." At the risk of sounding descriptive and not proposing any mechanism for change, I believe the researcher's time in preparing resubmissions is every bit as valuable as that of the reviewer and administrative apparatus. What is the alternative to resubmission, assuming that there is a relatively favorable impression of the proposal?

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Quantal Fluctuations: Correction

We have discovered misstatements in our report (*1*) "Intrinsic quantal variability due to stochastic properties of receptor-transmitter interactions" (27 Nov. 1992, p. 1494). First, the description of the Monte Carlo model incorrectly states that the diffusion constant used in the simulations was in the range of 0.5 to 1.0 × 10^{-6} cm^2 s^{-1}, when it actually was one order of magnitude greater (0.5 to 1 × 10^{-5}). Second, the time step was not 1 μs, as stated; the computer program we used automatically adjusted this variable to avoid unacceptably large transition probabilities such as would have occurred with the kinetic parameters used in those simulations. Typically, this resulted in a time step one order of magnitude smaller than what was reported. In addition, the inhibitory postsynaptic potentials illustrated in figure 3, B to D, of the report (p. 1496) were composite responses and not quantal events in the goldfish. The quantal responses are quite smaller. Further analyses of quanta isolated in tetrodotoxin (TTX) demonstrates they have a similar relationship between the mean and variance waveforms (Fig. 1). Thus, although the analysis illustrated in figure 3, B to D, is for larger responses, the results remain valid.

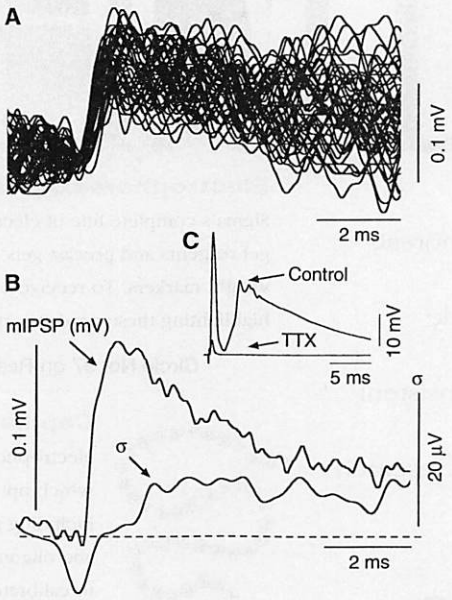

Fig. 1. Increased variance during the falling phase of quantal inhibitory responses. Intracellular recordings from the M-cell soma of adult goldfish, in the presence of TTX. (**A**) Superimposed miniature inhibitory postsynaptic potentials (mIPSPs, n = 47), demonstrating increased fluctuation in the waveforms during their decay phase. mIPSPs are depolarizing because the recordings were obtained with a KCl containing microelectrode. (**B**) The mean and standard deviation (σ) waveforms from the same data set. As responses are aligned at onset, σ at that time point is less than that of the background (dashed line), but exceeds it during the decay of the mean mIPSP. (**C**) TTX blocked medullary impulses, as demonstrated by comparing the antidromic action potential and the subsequent collateral IPSP recorded in the control condition with the same responses obtained at the time the mIPSPs were collected. Noise data were digitized with a 25 kHz sampling rate and filtered at 1 kHz with a second-order Bessel filter.

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1. D. S. Faber, W. S. Young, P. Legendre, H. Korn, *Science* **258**, 1494 (1992).

■

Authorship Disputes

Jocelyn Kaiser ("Commission proposes new definition of misconduct," News, 29 Sept., p. 1811) says the Commission on Research Integrity may recommend that ORI (the Office of Research Integrity) and institutions "no longer dismiss plagiarism cases involving collaborators as mere authorship disputes." The Commission's intent is better than its logic. There are no "mere" authorship disputes.

Authorship disputes concern stealing. A complaint is made that an author has presented someone else's work as his or her own, an act of intellectual theft. Complaints may be minor—little may have been stolen—or unfounded, but with the term "mere authorship disputes," the illusion is created that no theft is involved. The Commission should not have repeated this canard.

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Letters to the Editor

Letters may be submitted by e-mail (at science_letters@aaas.org), fax (202-289-7562), or regular mail (*Science*, 1333 H Street, NW, Washington, DC 20005). Letters are not routinely acknowledged. Full addresses, signatures, and daytime phone numbers should be included. Letters should be brief (300 words or less) and may be edited for reasons of clarity or space. In October 1995, our previous policy of consulting with all letter authors before publication was discontinued.

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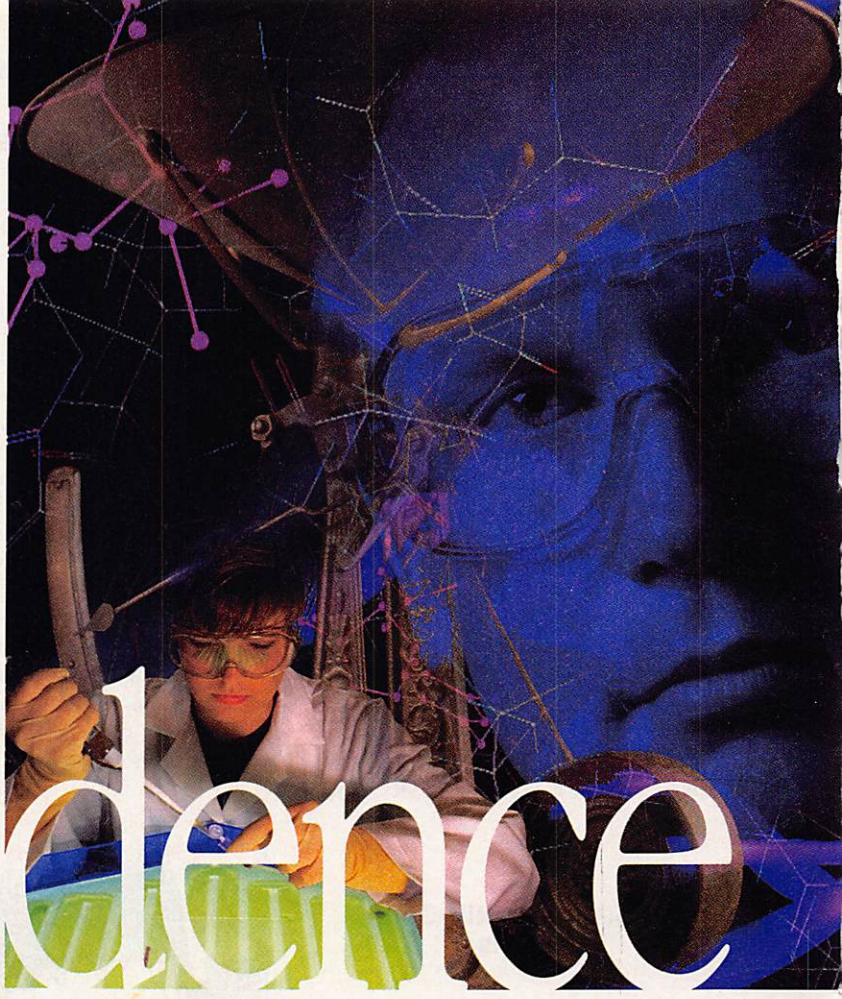

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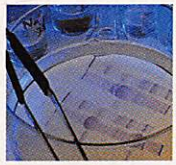

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edited by JOCELYN KAISER

NCI AIDS Budget Under Microscope

Once again, the intramural AIDS research program at the National Cancer Institute (NCI) is on the griddle. This time, the chef is the Gay Men's Health Crisis, a New York–based AIDS advocacy group, and the main dish is a spicy 35-page report that details—for the first time—how NCI divvied up the $172 million it allocated to intramural AIDS research in 1994. "It's a very scholarly report," says Alan Rabson, NCI's deputy director.

The analysis includes a breakdown of how much AIDS money each NCI principal investigator (PI) received last year and how many citations each one has on the AIDSLine papers database. More than $5.5 million was spent on researchers who have no citations whatsoever on AIDSLine.

Part of the problem, as a critique of NCI known as the Bishop-Calabresi report pointed out last spring (*Science*, 26 May, p. 1121), is that many investigators' work has AIDS applications but is not primarily focused on the disease. One glaring example is John Schiller, a respected papillomavirus researcher (see table). Rabson says NCI is now cutting back Schiller's AIDS dollars.

Another eyebrow-raising finding is that NCI's intramural program sucks up 81% of the institute's $212 million AIDS budget. That, too, will soon change, says Rabson, in part because three of the 10 best funded researchers have since left NCI. "We're hoping to move considerable amounts of that money to extramural grants," says Rabson.

TOP NCI AIDS PIs IN FY1994

PI	Lab	Budget (millions)	AIDS Cites
W. Blattner	Viral Epid.	$3.63	59
S. Tronick	Cell. & Mol. Biology	$1.95	14
G. Franchini	Tumor Cell Biology	$1.84	75
S. O'Brien	Viral Carcinogenesis	$1.72	11
R. Gallo	Tumor Cell Biology	$1.65	518
J. Lautenberger	Molecular Oncology	$1.47	9
G. Shearer	Exper. Immun.	$1.37	87
J. Schiller	Cellular Oncology	$1.10	0
P. Pizzo	Pediatrics	$1.05	88
F. Rusceti	Leukocyte Biology	$0.98	9

SOURCE: GAY MEN'S HEALTH CRISIS

Biomedicine Loses a Friend in Congress

Next year is shaping up to be a watershed in Congress, as 12 senators and 15 representatives have said they will not run again. Included in this departing flock is a strong supporter of basic biomedical research, Senator Mark Hatfield (R–OR), now chair of the Senate Appropriations Committee. Hatfield, 73, announced on 1 December that he will retire and move to Oregon's coast.

Often at odds with his own party's agenda, Hatfield never in his five Senate terms voted for a defense authorization bill. Meanwhile, he's given a "lifetime of support to biomedical research," says a longtime congressional staffer. The aide believes that "support for the National Institutes of Health is broad but thin" in Congress, and it isn't clear who, if anyone, will become NIH's next "white knight" in the Senate. Another aide notes that Hatfield played a critical role this year in pushing the Senate budget process to allocate $1.5 billion more than the House wanted in the bill that includes funding for NIH.

Fossil Bill Has Scientists Fuming

Legislation that would allow amateur and commercial fossil collectors to dig on federal lands is stirring up dust among paleontologists, who argue that unskilled collectors will destroy sites and plunder them of fossils that belong in museums.

Only academic paleontologists and their trainees can obtain permits to collect fossils on federal property. But the Fossil Preservation Act, soon to be introduced by Representatives Tim Johnson (D–SD) and Joe Skeen (R–NM), would permit anyone to take fossils from the surface using hand tools in areas not containing "scientifically unique" fossils. It would also allow amateur and commercial collectors to excavate on federal land if they signed an agreement with an overseeing research institution and promised to turn over to the area's manager any "scientifically unique" find. Marion Zenker of the American Lands Access Association (ALAA) says the bill would curb "the horrendous loss of fossils ... due to weathering."

Paleontologists, however, have been blasting the bill on the Internet, saying that while there's a need for a new fossil law, the proposal lacks tough penalties and provisions for strong oversight. Jerry Harris, a graduate student at Southern Methodist University, warns that amateurs without proper training could damage specimens or sites. And David Krause, president of the Society of Vertebrate Paleontology, says he has "very grave concerns" that the bill will allow fossils that should be in museums to go instead to private collections. The term "scientifically unique" is murky, he says, and "significant fossils are not necessarily unique." A staffer for Johnson says he is "cautiously optimistic" that the bill will pass this Congress. The ALAA also hopes for a companion bill in the Senate.

TED FREMD/YALE COLLECTION

Prehistoric loot? Law would affect protection of fossils such as this, from a hoofed mammal in Oregon.

Defense Bill Backs EPSCoR, Hits Labs

Tucked into the $243 billion defense spending bill that President Clinton reluctantly signed last week are two provisions that inadvertently line up with recent advice from the National Academy of Sciences (NAS) to make peer-reviewed, academic research the government's highest priority (*Science*, 1 December, p. 1430). One preserves a $20 million program that gives university scientists in have-not states seed money to vie for federal grants, and the other slashes $90 million from the military's $1.1 billion pot for its contract labs.

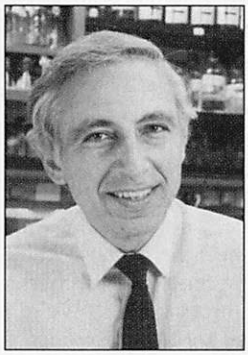
The state aid plan, called the Experimental Program to Stimulate Competitive Research (EPSCoR), is part of an $80 million multiagency effort that began in 1978. Congress overrode the Defense Department's attempt to kill the program, keeping its current level of funding. "We did pretty well considering the rest of the budget," says Stu van Scoyoc, a lobbyist for EPSCoR states. But the holiday season is a lot bleaker for scientists at the Pentagon's R&D labs: Congress cut $90 million from that account for the third straight year. One of the largest, the $275 million Lincoln Laboratory in Lexington, Massachusetts, is bracing for a cut of $20 million to $25 million, says a spokesperson. That's on top of a 21% decline in staff, to 2200, since the early 1990s.

While the defense bill helps parts of academia, it ignores an NAS plea to hold the line on science and technology funds, cutting basic research by 4%, to $1.17 billion. And by giving defense $7 billion more than the Administration requested, the measure puts the squeeze on pending bills that fund civilian research.

Elusive HIV-Suppressor Factors Found

Three factors, secreted by immune system cells, that apparently work in concert to suppress HIV replication have been identified after a long search; a fourth molecule may also be involved

ROME—HIV is infamous for outfoxing the immune system and ultimately destroying it. But researchers have long had hints that the immune systems of some HIV-infected people who don't progress to AIDS can outfox the virus. One of the strongest leads on what lies behind this ability has involved white blood cells known as CD8s: In 1986, virologist Jay Levy and researchers at the University of California, San Francisco, first reported that these cells can suppress the replication of HIV. And 3 years later, Levy and his colleagues demonstrated that the CD8s accomplished this feat by secreting an unidentified "factor" that potentially suppresses HIV's ability to copy itself. Yet no one had been able to identify this factor. Now, however, investigators have homed in on a trio of previously known immune molecules that they believe work in concert to produce the effects Levy first observed. If they're right, they not only will have solved a long-standing scientific mystery, they also will have paved the way for possible new treatments and new strategies to evaluate preventive vaccines.



NATIONAL CANCER INSTITUTE

Key factors. Team leaders Robert Gallo *(top)* and Paolo Lusso.

The new work comes out of the former National Cancer Institute lab of Robert Gallo, the co-discoverer of HIV and director of the new Institute of Human Virology at the University of Maryland. As Gallo briefly revealed at an AIDS meeting here last week,* a team headed by himself and Paolo Lusso, now at the San Raffaele Scientific Institute in Milan, Italy, found that three closely related polypeptides involved in inflammatory responses work together to produce the Levy effect. Known as RANTES, MIP1-α, and MIP1-β, these so-called chemokines shut down production of a broad range of strains of HIV-1, HIV-2, and SIV (the simian AIDS virus) that were growing in laboratory cultures. "There is something happening here," says Anthony Fauci, chief of the National Institute of Allergy and Infectious Diseases. "There is no question that this is door-opening time."

Gallo's abbreviated discussion of the finding followed a decision by *Science* to lift the embargo on a paper detailing the work, which will be published in the 15 December issue. *Science* took this action to coincide with the publication in the 7 December issue of *Nature* of a scientific correspondence, which *Nature* rushed into print, reporting a different—and apparently less powerful—HIV-suppressing factor that is also secreted by CD8 cells. Rumors of the Gallo-Lusso work were already widespread, and *Science* editors decided that it could potentially create confusion on a topic with clinical implications to keep the paper under embargo for another week.

For many scientists attending the Rome meeting—a small number of whom had been given embargoed copies of the *Science* paper—Gallo's news overshadowed the other highlights of the gathering, which included a dinner at the lavish palace of the Princess Pallavicini and a surprise visit by Mother Theresa. "The paper seems to be very convincing," says immunologist Sergio Romagnani of the University of Florence. "It provides direct evidence that the CD8 suppression of HIV is mediated by these chemokines."

Despite the considerable enthusiasm for the Gallo group's discovery, there is at least one voice of dissent—and it's coming from Levy. "I'm gratified that people are turning to look at what we have been working on," says Levy, who was reached by *Science* while on vacation in Brazil. "But if they say it's the factor I've been working on for the past 5 years, they've got the wrong ones." Levy, who says he had already screened for RANTES, MIP1-α, and MIP1-β as candidates for the elusive factor and ruled them out, nevertheless agrees that these chemokines may have promise as anti-viral therapy for HIV-infected patients.

Gallo says he is convinced that he and his co-workers have indeed identified Levy's factor, because they followed Levy's published protocols in preparing the CD8 cells used in their own experiments. What's more, as their paper shows, they abolished all of the HIV suppressor activity by adding to their cell cultures antibodies that specifically "neutralized" these three chemokines. Yet Gallo maintains that it "may be beside the point" whether these chemokines are the Levy factor. "This paper describes a major anti-HIV activity of CD8 cells," he says.

The brief scientific correspondence published in this week's issue of *Nature* by Reinhard Kurth, director of the Paul Ehrlich Institute in Langen, Germany, and his colleagues adds another layer of complexity to the picture. The Kurth group reports evidence that interleukin-16 (IL-16), a recently identified chemical messenger known as a cytokine, also suppresses HIV replication.

Kurth has long been investigating why African green monkeys do not become sick from SIV, which can quickly cause AIDS in Asian monkeys. The Levy factor had been a main suspect. Kurth's correspondence reports the isolation of nearly identical IL-16 molecules from humans and African green monkeys, both of which suppress HIV replication at relatively high concentrations in cell cultures. Levy also rejects this cytokine as a candidate for his factor, however, saying that his group eliminated IL-16 in recent experiments.

Fauci, who wrote a commentary in *Nature* to accompany Kurth's letter, told *Science* that it's possible that the Gallo group's factors and IL-16 may all play a role in HIV suppression. However, he said he found the Gallo group's results especially convincing because "Gallo is getting really impressive suppression at low concentrations [of chemokines], and in the Kurth paper the concentrations are much higher." Fauci adds: "They are both important papers. ... It is too much to ask that there is going to be one factor to the exclusion of all others."

The factor hunt

Levy first had a notion that there was something different in the CD8 populations of some HIV-infected people nearly a decade ago. CD8 cells are famous for acting like heat-seeking missiles that are directed at cells infected with HIV or other pathogens. But in 1986, Levy reported in *Science* another

* "AIDS and Related Diseases," Italian National Association Against AIDS, Rome, Italy, 30 November to 4 December 1995.

curious property of a subset of CD8 cells: They could strongly inhibit replication of HIV. And in 1989, he demonstrated that the inhibition was due to a soluble factor secreted by the CD8s. Interestingly, Levy found that the level of this HIV-suppressing activity appeared to decline as HIV-infected patients progressed to full-blown AIDS.

Yet for a number of years, Levy's work was regarded with widespread skepticism. "Years and years went by with no one paying attention," says Max Essex, head of the Harvard AIDS Institute at the Harvard School of Public Health. "My impression is that people didn't really believe it at all." The race for the factor began in earnest only within the last 2 years, when several leading AIDS researchers began to focus on the problem.

Lusso suggests that the sudden interest in the Levy factor was sparked by the modest results from AZT and other anti-viral therapies, as well as dimming hopes that there would be an anti-HIV vaccine any time soon. "I guess it was at this point that scientists started to realize that a simple, straightforward approach to therapy or vaccines was going to bring a lot of disappointment," he says.

Lusso says he believes his group succeeded where others have come up empty-handed because of two technical breakthroughs. One is the in vitro culture of a line of CD4 white blood cells—the main cell that HIV infects and destroys—called PM1. This PM1 line is easy to infect by fresh isolates of HIV taken directly from infected patients, as well as by strains that preferentially infect immune cells called macrophages. And, as compared with HIVs that have been passed through generations of laboratory cell cultures, these strains are the most sensitive to the CD8 suppressor factor.

The other critical technical advance, says Lusso, is a new immortalized CD8 cell line developed by the Gallo group that pumps out high concentrations of the three chemokines. With enough material to work with, the team was able to concentrate the polypeptides in crude fractions and then further purify them using high-performance liquid chromatography.

When the team sent the compounds to an outside lab for analysis of their amino acid sequences, they could not believe their luck: The sequences matched those of RANTES as well as MIP1-α and MIP1-β. But the critical experiments remained to be done. To prove that the three chemokines were in fact responsible for the suppressor effect, the group had to demonstrate two key points: First, that pure versions of the compounds could suppress viral replication when added to HIV-infected CD4 cells; and second, that the suppressor effect of supernatants of CD8 cells taken from HIV-infected individuals could be eliminated by the action of neutralizing antibodies directed against the chemokines.

DAVID POWERS

The Levy factor. Jay Levy provided first evidence for a suppression factor secreted by CD8s.

The results of these experiments led to yet another eye-opener. The team found that although RANTES potently inhibited HIV production, MIP1-α and MIP1-β were somewhat less powerful. More troubling still, when antibodies to the three chemokines were added individually to the CD8 cell supernatants, they had very little effect. But when the researchers hit on the idea of using all three chemokines at once, they struck pay dirt. In the paper's most dramatic result, the researchers discovered that when they added neutralizing antibodies to all three chemokines at once, they were able to abolish all of the HIV suppressor activity of the CD8 supernatants.

Fourth factor. Reinhard Kurth found IL-16 also appears to suppress HIV.

Indeed, it is the group's antibody work that has most convinced other researchers of its validity. Immunologist Hans Wigzell, president of the Karolinska Institute in Stockholm, Sweden, says the results are "highly credible because they are at low, seemingly physiological, concentrations. From the point of view of scientific curiosity, it is a milestone. The hard thing is next—to see the clinical relevance in patients."

Moreover, the antibody studies may help to explain some of the discrepancies between the Gallo-Lusso paper and the Kurth group's letter in *Nature*, as well as at least some of the objections raised by Levy. Kurth told *Science* that in preliminary experiments he tested both RANTES and IL-16, but found that "RANTES didn't work and IL-16 did work." However, Kurth says, he tested RANTES only in a T cell lymphoma cell line infectible by HIV—a test system unlikely to be as sensitive as the Gallo group's specially designed PM1 cell line—and did not add the other two chemokines at the same time. "We will now put all four factors into the test system," he says. "Nobody at this stage knows to what extent the different factors contribute to HIV suppression."

Likewise, in the process of screening for known immunologically active molecules over the past several years—which involves the use of neutralizing antibodies to test for their activity—Levy says he cannot recall whether he tested for RANTES, MIP1-α, and MIP1-β at the same time. A number of researchers contend that if Levy only tested one chemokine at a time he might easily have concluded that they were not effective in suppressing HIV. Nevertheless, Levy maintains that he has seen no correlation between individual concentrations of these chemokines and HIV suppressor activity.

Another reason Levy doubts that these chemokines are the factor he has been hunting for is that they do not appear to act directly on the process of viral RNA transcription—the mechanism he has postulated as the primary explanation for how his factor suppresses HIV replication. Gallo and Lusso note that their factors do inhibit RNA transcription, but they believe that the block may come at an earlier stage of the virus' life cycle, before transcription begins.

"Levy has not proven that it is at the transcriptional level," Gallo says, although he concedes that Levy's results are "compatible" with transcriptional blockage. But a number of researchers say they are most intrigued by the fact that the three factors act together in some way. "It's extremely interesting," says Antonio Siccardi, coordinator of AIDS research programs at the San Raffaele Institute in Milan. "It makes me think about some kind of synergy."

However the factors exert their HIV-suppressing actions, AIDS researchers say these studies should be followed up quickly. "If I was the typical pharmaceutical company, I'd be looking at all of these," says Essex. "I wouldn't be sitting on my butt making one more derivative of AZT." At the moment, of course, the ability of these compounds to inhibit HIV has only been demonstrated in the test tube. And Gallo, for one, is circumspect about whether the chemokines will lead to new therapies. "There is no reason to believe that this is the Holy Grail," he says. Still, he and Lusso will soon launch toxicology studies in animals, with the ultimate goal of beginning human clinical trials at Gallo's new institute in Baltimore and at the San Raffaele Institute in Milan.

Marc Girard of the Pasteur Institute in Paris, a leader of the French AIDS vaccine effort, says it's too early to say whether these chemokines will have practical applications. But he, too, is optimistic. "You open a new window in the wall, and then you have to look through the window to see what you can see," says Girard. "There's a whole new landscape out there." And it's a landscape that many researchers are sure to begin exploring quickly.

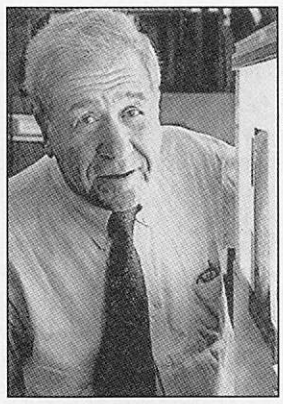
–Michael Balter

BREAST CANCER

Reanalysis Confirms Results of 'Tainted' Study

Women with breast cancer received mixed reports from the research establishment in the past week. The good news is that lumpectomy (minor surgery) has been shown to be just as much a lifesaver as mastectomy in treating small tumors. This result was first announced a decade ago, on the basis of a major clinical trial, but it had been clouded by reports that some data in the trial were tainted by fraud. Now a detailed reexamination of the untainted data leaves no doubt that the original conclusion was valid. But a major, and surprising, disappointment has come from another study: A long-term clinical trial of tamoxifen—a drug used to treat breast cancer—has been canceled, and physicians have been notified that tamoxifen appears to lose therapeutic value after 5 years' use (see box).

The data comparing lumpectomy with mastectomy come from a huge study of more than 2000 patients, orchestrated by Bernard Fisher of the University of Pittsburgh. His 89-center collaborative group—known as the National Surgical Adjuvant Breast and Bowel Project (NSABP)—found in studies published in 1985 and 1989 that lumpectomy, lumpectomy with radiation, and mastectomy were all equally effective in extending the lives of women with small (4 cm or less) tumors. The results have now been shown valid for an average patient follow-up of 12 years. Although cancer reappeared at a high rate among women who had lumpectomy alone—about 35%, compared to 10% among women who underwent mastectomy or lumpectomy-plus-radiation—it did not significantly increase mortality in this analysis.



TERRY CLARK/NYT

Untainted. Fisher says new analysis vindicates his work.

After churning out such data for 18 years, NSABP ran into trouble in 1994. A surgeon at one of the participating hospitals—St. Luc's in Montreal—was discovered to have falsified the records of six patients in the lumpectomy study, also known as the B-06 trial. The *Chicago Tribune* ran an exposé of the case, and Representative John Dingell (D–MI) held an investigative hearing at which he raked Fisher and the National Cancer Institute (NCI) over the coals. Because the validity of NSABP's data had been questioned, the government conducted a massive audit of B-06 patient records. Fisher and NSABP staffers also reanalyzed the data independently. After 18 months of fencing among the NCI, Fisher, and the *New England Journal of Medicine*—which had published the original study and was upset not to have heard about the tainted data sooner—*NEJM* published a reanalysis last week. It also published an audit by NCI and a 36-trial meta-analysis by a large collaborative group confirming Fisher's initial results.

"We are very pleased to see this come out at last," Fisher said in a telephone interview. NCI's audit of the study, by Michaele Christian and colleagues, found discrepancies in the NSABP data but judged them to be "uncommon" or not significant enough to affect the study's conclusions. The NCI team audited only 86% of the patients in the study because, a staffer says, it would have been prohibitively expensive to track down every last one. Of the 1554 audited cases, NCI reported that 4.4% contained "at least one discrepant item," and 7.3% had one or more unverified item.

The most striking defect, according to biostatistician John Bailar of the University of Chicago, writing in the same issue of *NEJM*, was the failure of NSABP physicians to "be meticulous about informed consent" before patients were assigned to a treatment group. Only two thirds of the patient files contained consent forms that had been signed before surgery. Documented consent was obtained after surgery in 210 cases; consent forms were undated in 137 cases; and consent was not verified in 71 cases. Bailar wrote that he found this record "unjustifiable."

Asked to rate the trial today, Fisher said, "It was impeccably run. … It emancipated the scientists and clinicians from concepts that governed breast cancer surgery for the major part of this century."

–Eliot Marshall

Long-Term Tamoxifen Trial Halted

The demand for tamoxifen, a synthetic hormonelike drug, has been growing steadily since the 1980s, when it was found to help prevent the recurrence of breast cancer. The drug locks onto estrogen receptors in tumors that contain them, blocking the growth stimulus that estrogen provides. But last week, preliminary results of a major clinical trial indicated that this effect may fade with prolonged use; in fact, the findings prompted concern that long-term tamoxifen use may even increase cancer risks for some women.

As a result, managers of a major cancer research project—the National Surgical Adjuvant Breast and Bowel Project (NSABP) in Pittsburgh—immediately canceled a long-term tamoxifen therapy experiment known as the B-14 trial. The National Cancer Institute (NCI) also sent a bulletin to 22,000 physicians on 30 November advising them that there is "no advantage" for women with "node-negative, estrogen-receptor-positive breast cancers" to take tamoxifen for more than 5 years. NCI added that there is a "troublesome possibility" that using tamoxifen for more than 5 years "might actually be detrimental."

"This is very big news; it's a surprising result," says Lauren Schnaper, an oncologist who directs a breast cancer study involving older women at the University of Maryland, Baltimore. She had planned to give tamoxifen to patients in one arm of her study for an indefinite period. The trial is now being redesigned. But while patients in clinical trials will get the new warning on tamoxifen, it may be more difficult, she says, to change use by physicians not in trials. "Many people in this country feel that tamoxifen is just a wonder drug," Schnaper says. According to one NCI staffer, as many as 1 million U.S. women may be taking tamoxifen, perhaps 20% of them for longer than 5 years.

The news on tamoxifen comes from a special review conducted last month by members of NSABP's data and safety monitoring committee. They found that 92% of those who had taken tamoxifen in the B-14 trial for 5 years were disease-free for 4 years after they stopped taking it, versus 86% in the group that had taken it longer than 5 years. The long-term group had six new endometrial cancers compared to just two in the 5-year group; six new contralateral breast cancers versus five; and 12 other new primary cancers versus nine. These findings matched results from a soon-to-be published Scottish study.

Jeff Abrams, the NCI official who monitors NSABP, says animal studies have suggested theories why tamoxifen doesn't work well after 5 years—such as that tumors exposed to the drug for long periods adapt and begin responding to it as they do to estrogen. But Abrams notes it will take more basic research to get the complete answer.

–E.M.

PLANETARY SCIENCE

Clementine 2 to Size Up an Asteroid

If all goes according to plan, the world will get its first close-up look at a potential planetary assassin in 1998, when a nimble, low-cost spacecraft will rendezvous with three asteroids whose paths cross Earth's orbit.

Nestled unobtrusively in the $243 billion defense budget for 1996, which became law last week, is the first $20 million installment for a follow-on mission to last year's Clementine 1, a $100 million probe that used cheap, sophisticated sensors to map the moon. Clementine 2 scientists from the Lawrence Livermore National Laboratory (LLNL) and the Naval Research Laboratory (NRL) plan to apply the same penny-pinching strategy to deliver "our first close-up look ever at an Earth-crossing asteroid," says Eugene Shoemaker of the U.S. Geological Survey in Flagstaff, Arizona, the project's chief science adviser. "I think we're going to have a very exciting mission."

The Pentagon's Ballistic Missile Defense Office (BMDO) sponsored the first Clementine, viewing it as a test bed for sensor technology. Since then, BMDO has dropped out, but the Air Force Space Command has snapped up the program. Shoemaker, a co-discoverer of the comet that slammed into Jupiter last year, says the Air Force has adopted "planetary defense" as a new mission area, "and Clementine is definitely part of it."

The spacecraft will cost between $100 million and $120 million, a fraction of the cost of a traditional NASA mission. NRL scientists have already begun searching for asteroids that will cross Earth's orbit in late 1998, the target launch period, hoping to find three that can be visited in a single mission. LLNL scientists will design low-cost, light-weight sensors and cameras that will map the asteroids from a distance of a few hundred kilometers.

The spacecraft will also be armed with three probes, one per target asteroid. These impactors will photograph the asteroids at close range; then they will slam into their targets. The mother ship will scrutinize the resulting dust and fragments for clues to the asteroids' composition and structure.

To keep launch costs down, all that technology will have to fit in a package weighing just 180 kilograms, 40 kilograms less than the first Clementine probe. That leaves just 10 kilograms for each camera-equipped probe. "It's tough, but it's doable," says Ed English, a project scientist at LLNL.

To scientists who advocate developing a defense against asteroids that threaten to collide with Earth, notably LLNL's Edward Teller, it's only a first step. Clementine 2 "is the only realistic way we can get information on the mechanical properties of these objects," says Shoemaker. The next step, say Shoemaker, Teller, and others, is to figure out how to destroy them.

–Jonathan Weisman

Jonathan Weisman is a science and defense writer at The Oakland Tribune.

JAPAN R&D BUDGET

Public Spending Falls for First Time

TOKYO—Government R&D spending in Japan declined last year for the first time in 40 years, according to new figures released last week. The drop comes at the same time corporate spending on research—which makes up almost 80% of the country's total R&D investment—declined for the third year in a row.

Officials say the main reason for the 1.6% drop in government spending, to $29.2 billion, was a large economic stimulus package that boosted research by almost 10% in 1993. The upward trend is expected to resume in 1995 because another large supplemental spending bill approved this fall will add billions of dollars to ongoing research programs. But scientists and policy-makers say that a steady and predictable increase in base budgets would be better for research planners than a sudden windfall from an economic stimulus package. Scheduled increases would also be more in tune with a campaign to double government R&D spending by 2000.

The latest figures on Japan's research expenditures come from an annual survey, including more than 10,000 private corporations, 1500 research institutes, and 2500 universities, conducted by the Management and Coordination Agency. The drop in government spending on research during fiscal year 1994, which ended 31 March 1995, was the first recorded dip in the history of the survey, which began in 1953. Private spending dropped to $106.6 billion, a 1% decline from 1993 and a 5.2% drop from a peak of $112.6 billion in fiscal year 1991.

Shin Aoyama, director of the research division of the Science and Technology Agency's (STA's) policy bureau, says that the 1993 supplemental spending packages added $5.5 billion to base research budgets. In contrast, new spending packages for 1994 boosted research by only $100 million, and the drop in supplemental spending outweighed a small increase in base budgets.

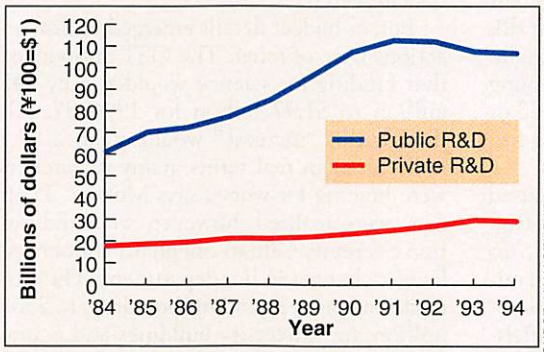
This two-steps-forward, one-step-back pattern is likely to be repeated in 1995–96. With the nation's economy still in the dumps, the government this year enacted two more supplemental spending packages, including an additional $6.8 billion for research-related equipment and infrastructure. But Aoyama says it is unlikely that such mammoth supplemental spending packages will be approved year after year.

Although scientists welcome the shots in the arm from the supplemental spending bills, they would prefer to see a larger rise in base budgets. "For continuity, we'd rather see that level of spending in the original budget, instead of the supplemental budgets," says Masao Ito, a neurobiologist at the Institute of Physical and Chemical Research outside Tokyo and president of the influential Science Council of Japan. Ito is also a member of the Council for Science and Technology, an advisory body to the prime minister, which in 1992 recommended that the government double its spending on research. The cabinet adopted the policy later that year but declined to set a target date, specifying only that it be "as soon as possible."

Ito says support for the doubling to occur by 2000 is growing within government research agencies. Earlier this year the STA, the Ministry of International Trade and Industry, and the Ministry of Education, Science, Sports, and Culture began including the date in position papers, although it is not official government policy. The biggest holdout is the Ministry of Finance, which is trying to reverse a growing budget deficit exacerbated by the continuing recession.

Within the commercial sector, Japanese companies are continuing to expand R&D activities overseas even as they rein in domestic spending. The management agency's report notes that expenditures outside of Japan, mostly in applied research, hit an all-time high of $140 million.

–Dennis Normile



In step. Both government and corporate R&D spending in Japan dropped last year, continuing an industry trend.

RADIO ASTRONOMY

Astronomers Protect Endangered Bands

Relegation to a footnote in a document is not usually anything to boast about. But radio astronomers are treating a handful of footnotes in a paper produced at the World Radio Conference held last month in Geneva, Switzerland, as a major achievement. The footnotes ask governments to protect certain bands—especially the methanol line at 6668 megahertz—for exclusive use by astronomers. In a world where the radio spectrum is ever more crowded by broadcasting, satellite, and telecommunications companies and navigation services, this is no small issue.

Researchers had gone to the conference, sponsored by the United Nations' International Telecommunication Union (ITU) to coordinate the allocation of radio frequencies, to fight for their rights to the methanol band. They use the line, discovered in 1991, to observe newly forming stars in gas clouds deep in space: The stars excite methanol molecules, which amplify radio waves. And the *Radio Regulations* document, produced at the Geneva conference and signed by representatives of 140 countries, contained in Section 5 that all-important footnote. "The single most important achievement was the protection of the methanol line," says Klaus Ruf of the Max Planck Institute for Radio Astronomy in Bonn, Germany. "These footnotes are not strongly worded," says Willem Baan of Arecibo Observatory in Puerto Rico, who led the astronomical delegation, "but generally they are respected, although they are not legally enforceable."

The fight for bandwidth is an ongoing struggle. Only one band is formally reserved for astronomers: the hydrogen line at 1420 megahertz, which is used for detailed study of galactic structures and redshifts. "We have to be vigilant in order to protect what we have," says Baan. "It is a continuous battle, and it seems that we are slowly losing." For instance, astronomers are often asked to share bands with telecommunications companies, but the two uses are incompatible, says Hans Kahlmann, director of the Westerbork Radio Observatory in the Netherlands. Telecoms "are used to signal-to-noise rates of 20 dB, while we deal with incredibly weak radio sources and incredibly sensitive receiving systems with signal-to-noise rates of –60 or –80 dB. We can't even think of sharing frequencies," he says.

Frequency overlap has already caused trouble with the methanol line, says Ruf. German telephone services have begun to use the methanol band for ground-based microwave links. "Every time we want to observe in it with our radio telescope in Effelsberg, I have to write or call the German PTT and ask them to close down fixed links close to the telescope, and usually they comply."

The ITU meetings are the place where astronomers have to make their case. And in Geneva, "we lost less ground than we expected," says Kahlmann. In addition to the methanol line, they also obtained footnotes urging governments to protect certain bands below 1 gigahertz, the 4990 to 5000 megahertz band, and the 1535 to 1540 megahertz band, all of which are currently subject to interference from satellite downlinks. The footnotes are important, Kahlmann, says, but it is up to communications authorities in each country to implement the recommendations, and astronomers would like stronger protection. "We hope to get ... exclusive rights for all these bands, like we have now for the hydrogen line, but we will have to fight for it," he says.

And going head-to-head against firms that might use these bands to make money isn't going to be easy. Astronomers would have a stronger position if they had applied for licenses for particular bands when the World Administrative Radio Conference in 1959 accepted radio astronomy as a communication service. But they didn't, and Kahlmann says that was a big mistake. Licenses—and their attendant rights—"would give us more clout in the negotiations," he says.

–Alexander Hellemans

Alexander Hellemans is a science writer in Amsterdam, the Netherlands.

U.K. BUDGET

Equipment Funds Suffer Heavy Cut

LONDON—The British government dealt a cruel blow to researchers last week following the annual budget statement to Parliament by Chancellor of the Exchequer Kenneth Clarke. Clarke's statement indicated that the six research councils will get a small increase, although much less than the rate of inflation. But when details were released later, they revealed that money for capital projects in universities would receive a huge cut. "Funding through the universities is the essential second leg of the dual support for research, which has been completely knocked away by these cuts," says physicist John Mulvey of the pressure group Save British Science.

Scientists had not expected a generous handout this year. All ministries are under severe pressure to cut spending as the Conservative government, deeply unpopular in the opinion polls, focuses on vote-winning measures before the next general election due within 15 months. There was also the ill omen of the Office of Science and Technology's (OST's) surprise move from the Cabinet Office to the Department of Trade and Industry last summer—seen as a sign of the government's waning commitment to basic research.

But, as budget details emerged, initial reactions were of relief. The OST announced that funding for science would rise by $45 million to $1.97 billion for 1996–97. Although this "increase" would mean a $7.5 million cut in real terms, many researchers were bracing for worse, says Mulvey. Their fears were realized, however, when Education Secretary Gillian Shephard announced budget changes in her department: They included a massive $160 million drop, to $365 million, for university buildings and equipment next year. This 30% cut will grow to 52% over 3 years as part of a larger package of higher education cuts. Science departments will be hit hard, as the money is a key source of funds for large items of equipment and for maintaining laboratories, says an official of the Committee of Vice Chancellors and Principals (CVCP).

The universities were quick to condemn Shephard's announcement. "With the level of capital investment in the universities in decline over the past decade, a 30% cut in one year is potentially extremely serious," says Derek Roberts, provost of University College London.

Shephard said that institutions would be expected to make up for capital losses by raising extra money from the private sector under a new scheme, the Private Finance Initiative (PFI), applied to many areas of government and extended to the higher education sector 8 months ago. But this only rubbed salt into the wounds inflicted by the funding cuts. The CVCP said that the PFI was still experimental and "neither robust nor streamlined enough to replace in time the capital which has been lost."

Roberts, whose college has received the largest investment by a company of any university in the U.K.—$75 million from the Japanese pharmaceutical company Eisai—believes the initiative may be inappropriate for universities. "The PFI may be sensible for schemes like road-building where investors can get a return through tolls or other charges, but universities are not a place to make money in this way," he says.

Trade and Industry Secretary Ian Lang, now responsible for science, told a Save British Science delegation last month that he would be a strong advocate for science and technology. "But the budget shows that he has failed the research base," says Mulvey.

–Nigel Williams

GLOBAL CHANGE

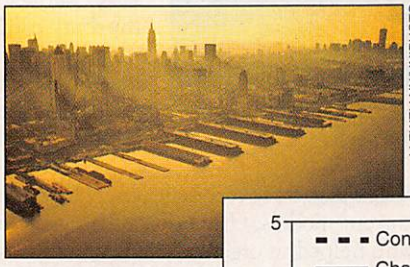
It's Official: First Glimmer of Greenhouse Warming Seen

A war of words among scientists and politicians warmed the air in Madrid last week, but the final words from an international panel of scientists assessing the state of climate science were cool and measured. Many uncertainties remain to be resolved, Working Group I of the Intergovernmental Panel on Climate Change (IPCC) concluded in a report* approved last week, but "nevertheless, the balance of evidence suggests that there is a discernible human influence on global climate."

A link between rising greenhouse gases, mainly carbon dioxide, and the global warming of the past century hasn't been proven unequivocally, notes atmospheric physicist Michael Oppenheimer of the Environmental Defense Fund in New York City, an IPCC contributor who was in Madrid. The scientists who prepared the relevant chapter are not yet certain that the half-degree warming isn't a natural fluctuation, notes Oppenheimer, in part because they don't know just how much climate varies on its own. These scientific uncertainties led to much debate over the exact phrasing of the conclusion—but the scientists' debate was tame compared to the wrangling among representatives of the governments assembled in Madrid. For example, oil-producing countries, mainly Saudi Arabia and Kuwait, argued long and hard for a retreat from the slightly stronger language of a draft report circulated in September (*Science*, 22 September, p. 1667).

Still, the final report announces that researchers now have a handle on the biggest problem in recognizing a human influence on climate—the disparity between the amount and geographic distribution of the warming predicted by climate models and the actual temperature records. The key was recognizing the cooling effect of the haze of fine particles that hangs over industrialized regions. This aerosol cooling alters the predicted "fingerprint" of the greenhouse effect by reducing the predicted warming and shifting its geographic pattern. More and more, the actual temperature record looks like this newly perceived fingerprint of human-induced climate change.

The first solid evidence that aerosols could be altering the signal of greenhouse warming came earlier this year when researchers reported data indicating that aerosols have cooled the climate of eastern North America, Europe, and south Asia in recent decades (*Science*, 12 May, p. 802). An aerosol-induced pattern of cooling, it seemed, must be superimposed over the greenhouse-warming pattern. So modelers fed both greenhouse gas increases and the effects of aerosols into the latest global climate models, which couple a model of the atmosphere to an ocean model complete with shallow and deep currents. They then used new, more sophisticated statistical techniques to gauge the similarities between the observed patterns and those predicted by the models. The first results seemed to show hints of the predicted greenhouse-plus-aerosols fingerprint (*Science*, 16 June, p. 1567).



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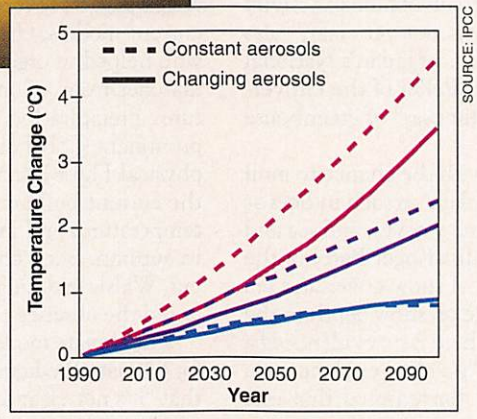


SOURCE: IPCC

Hazy outlook. In three different climate-change scenarios *(colors)*, increases in pollutant hazes *(inset)* moderate the warming.

By now, four next-generation studies from three research centers—Lawrence Livermore National Laboratory, Hadley Center for Climate Prediction and Research in Bracknell, United Kingdom, and the Max Planck Institute for Meteorology in Hamburg, Germany—have been published. All four studies, three of which focus on surface temperatures and one on temperatures aloft, show that over recent decades the observed spatial pattern of temperature change increasingly resembles the expected greenhouse-aerosol pattern. Says climate modeler Gerald Meehl of the National Center for Atmospheric Research: "It's really encouraging that when we add aerosols [to model simulations] we do start to see better agreement with the [climate] patterns we've already observed."

Other runs of the same computer models, done without increases in greenhouse gases or aerosols, suggest that this greenhouse fingerprint is not likely to be a chance fluctuation of the climate system. As predicted by the models, the natural variability of an undisturbed climate system looks different, statistically speaking, from the observed pattern of change. To bolster their quantitative studies a bit, the IPCC scientists also note a half-dozen different climate trends such as cooling of the stratosphere, increases in high-latitude precipitation, and rising sea levels that are all expected in a greenhouse-warmed climate—and have all been observed.

Still, the careful phrasing of the IPCC's conclusion reflects a range of opinion in the climate community. At one end is climatologist Tom Wigley of the University Corporation for Atmospheric Research in Boulder, Colorado, one of the four co-authors of the detection chapter. Speaking for himself last July at an IPCC session of an international meeting, Wigley made a stronger statement about human influence: "We can claim, with a high statistical confidence, to have identified an anthropogenic signal in the observed temperature record."

Climatologist Tim P. Barnett of Scripps Institution of Oceanography, also a co-author on the detection chapter, isn't so sure. He agrees that "we've moved closer to a point where you can say you've detected it for sure," but he emphasizes some of the caveats stated in the report itself. "I've worked with some of these coupled models, and they have some serious problems," says Barnett, including "flux corrections" that artificially keep them from drifting into unrealistic climate simulations (*Science*, 9 September 1994, p. 1528).

The models do behave well in the short run, he notes: "On annual to almost decadal time scales, we're finding the models do things that happen in nature," successfully predicting El Niños and their effects on climate, for example. But they haven't consistently reproduced longer records of climate change—for example, the temperature changes seen in this century, in which a sharp global warming was followed by a plateau from the 1940s through the 1970s and then by renewed warming. Barnett is concerned that the natural ups and downs in global temperature over such long periods may be larger than the models are suggesting, leading the more optimistic researchers to mistake natural fluctuations for greenhouse warming.

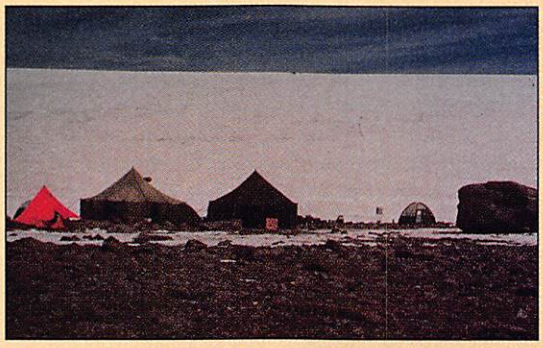
The report doesn't put that concern to

* Working Group I report of the Intergovernmental Panel on Climate Change. Contact Bruce Callander, bacallander@email.meto.govt.uk

Polar Regions Give Cold Shoulder to Theories

TSUKUBA, JAPAN—While a United Nations' panel surveying global temperatures sees the first signs of greenhouse warming (see main text), scientists searching for such evidence in the polar regions are coming up empty-handed. Climate models forecast that even minor warming from the buildup of greenhouse gases in the atmosphere would lead to a retreat of polar ice and snow cover, reducing the albedo, or reflectance, of the Earth's surface. That, in turn, would result in greater absorption of solar radiation, which would boost surface temperatures and cause a further retreat of snow cover. The result would be a greater temperature rise at the poles than in the equatorial regions.

But evidence of such a high-latitude warming is proving hard to find. Seasonal and regional variations in the polar regions make it difficult to separate suspected greenhouse signals from the noise of natural fluctuations. Last month, at a 4-day international conference here,* researchers agreed that detecting climate change in a single region is a lot trickier than was thought just a few years ago. "We are seeing changes, but whether these are the result of human activity or within the range of natural variability is not yet clear," says oceanographer Nobuo Ono, deputy director of Japan's National Institute of Polar Research. Adds Gunter Weller of the University of Alaska, Fairbanks, "we're seeing glimpses" of greenhouse signals, "but it's all very ambiguous."



LONNIE G. THOMPSON

Tibetan tales. Ice cores from the Qinghai-Tibetan Plateau supplement records from the poles.

The conference provided researchers with the chance to mull over the significance of a growing body of data on such indices as extent and thickness of land and sea ice, snow cover, and sea and air temperatures. Cryospheric data specialist Roger Barry of the University of Colorado reviewed surveys of snow cover and sea ice, including data recently made available on snow depth in the former Soviet Union. His conclusions: "There is no evidence of a recent warming trend" in these data and "no clear evidence of a trend" in the thickness of polar sea ice. Barry noted that one exception, a documented thinning of ice in the past 3 to 4 years in the East Siberian Sea, seems to be related to changes in air temperature patterns from an increase in the number of cyclones south of the sea. But it's not clear whether cyclone activity is itself an indicator of climate change, he said.

H. Jay Zwally, a glaciologist at Goddard Space Flight Center in Maryland, said his search for long-term trends in ice cover, based on a review of satellite-based remote sensing of polar ice, "has given ambiguous results." There have been tremendous variations since he began his observations in 1973, he noted, with sea ice cover in some regions fluctuating by as much as 30% over several years.

Confusing signals are also coming from ice cores, which researchers had once hoped would prove to be reliable records of historic climate variability. Ice-core specialist Roy M. Koerner of the Geological Survey of Canada pointed to problems arising from comparisons of data from the Greenland Icecore Project (GRIP) with other indicators of climate change in the Holocene period, which extends back 10,000 years. He suggested that GRIP's Holocene record of the levels of oxygen-18, an isotope trapped in the ice that is used to deduce the history of atmospheric temperatures, is not representative of the northern circumpolar region. "Ice sheets are so big they generate their own climate," he said.

Although Antarctic and Greenland ice cores provide good records for major climate changes, he said, they may not reflect the more subtle shifts that take place on shorter time scales. A record of these short-term variations is needed to establish the natural variability of interglacial periods.

Another expected signal of warming—the increased occurrence of abnormal weather—is also proving to be hard to pin down. Atmospheric scientist Hiroshi Tanaka of the University of Tsukuba's Institute of Geoscience has studied a few days in early 1989 when Arctic air temperatures switched from 10°C colder than normal to 20°C warmer than normal and found the sharp rise in temperature stemmed from a shift in wind currents above the Beaufort Sea. But "we don't know [whether there is] a trend," he said, because of a lack of historical data.

A second purpose of the conference was to try to reconcile observations with predicted results from current models. University of Illinois climatologist John Walsh, who helped to organize the conference, said he has found spotty matches from a comparison between observed Arctic temperatures, precipitation, and extent of sea ice and predictions from a prominent global climate model developed by Princeton's Geophysical Fluid Dynamics Laboratory. The model predicted that the current buildup of greenhouse gases would result in warmer temperatures and increased precipitation over the Arctic Ocean in autumn. Such changes were observed at the predicted levels, but, Walsh said, they occurred over land in the spring. Walsh also noted the absence of retreating sea ice, as the model implies.

But climate modeler Howard Cattle of Britain's Hadley Centre for Climate Prediction and Research defended the models, saying that "it's not clear to me that at this stage of the game you would expect to see a greenhouse signal." He noted that past models often were too simple, in particular overlooking the cooling effect of pollutant sulfate aerosols that tends to offset CO_2 warming (*Science*, 16 June, p. 1567). When the effects of aerosols are factored in, he said, they capture this century's warming trend, including a gradual rise in atmospheric temperatures into the 1950s, a leveling-off, and then a resumption of warming in the 1970s. However, the aerosol effect "pushes the [warming] curve back and may delay the onset of any observable signal," Cattle said. Another problem, he said, is that the models are not yet precise enough to accurately reveal the regional signals sought by polar observers.

Surrounded by all the unanswered questions, some researchers are wondering if the polar regions may not be the right place to look for early signs of global climate change. Indeed, Philip Jones, a climatologist from the University of East Anglia in the U.K., said that the extreme natural variability in the polar climate makes it more difficult to get a clear picture of trends than in other more stable regions. "The first specific region to look at may be the tropics," he said. Illinois's Walsh acknowledged that the idea is worth considering. But given the title of the conference, he said, such suggestions would have to be handled "in a subtle way."

–Dennis Normile

* Wadati Conference on Global Change and the Polar Regions, Tsukuba, Japan, November 7–10.

rest. But it does answer some criticisms raised by greenhouse contrarians. For example, critics had noted that climate models, even with aerosols included, did not predict the faster warming at night relative to daytime that was being observed (*Science*, 7 February 1992, p. 683). Now the report notes that climate modeler James Hansen of the Goddard Institute for Space Studies in New York City and his colleagues have gotten their model to reproduce the nighttime warming by combining rising greenhouse gases and aerosols and an increase in cloud cover—a phenomenon widely observed but poorly understood.

In another controversial area, the IPCC report rejects the claim that satellite monitoring shows just one quarter of the warming predicted by climate models. The problem with that claim, says the report, is that the greenhouse skeptics are making comparisons with the wrong model simulations—ones that had greenhouse gases rising more rapidly than has happened so far or that weren't designed to gauge temperature increases in the first place. The latest models, incorporating aerosols and a realistic rise in greenhouse gases, predict 0.08° to 0.30°C of warming per decade, which puts the satellite warming rate of 0.09°C per decade just within the predicted range. Even that comparison may not be meaningful, notes the report, given the variability of global climate from decade to decade and the short, 16-year satellite record.

Just what effect the emerging scientific consensus will have on policy-making remains to be seen. For one thing, even though scientists have spotted a human hand in climate change, they still can't say how large its effects will be in the future. "Our ability to quantify the magnitude of this effect is presently limited," cautions the IPCC report, which gives estimates that range from a mild 1°C warming by 2100 to a hefty 3.5°C.

Still, Oppenheimer believes that the new IPCC finding "increases the likelihood that a real schedule of real [greenhouse gas] emission reductions" will come out of current negotiations being conducted under the 1992 Climate Convention. For now, industrialized nations have made a commitment to reduce their emissions to 1990 levels by 2000, but they are not strictly bound to do so.

In the United States, where the Republicans in Congress oppose many environmental regulations, the IPCC report could affect the thinking of the more moderate among them, says Robert Watson, associate director for environment in the White House's Office of Science and Technology Policy. It may "give them pause and make them think very carefully. It supports the contention that this is an issue that merits serious action." But as Oppenheimer notes with some understatement, "The politics of the details will not be easy."

–Richard A. Kerr

EARTH SCIENCE

Missing Chunk of North America Found in Argentina

Half a billion years ago, North America was a lost continent. Present-day Africa, Australia, Antarctica, South America, and India had assembled into the supercontinent of Gondwana, but North America and a few smaller continental fragments were drifting on their own, and geologists have had few clues to their peregrinations.

"The big issue in the paleogeography of the world about [that] time ... was the geography of the biggest players in this game, North America and Gondwana," says geologist Ian Dalziel of the University of Texas, Austin. But at a meeting of the Geological Society of America last month in New Orleans, speakers described how a chunk of crust in western Argentina is turning out to be North America's calling card. Dropped off in western South America nearly 500 million years ago, it pins down the errant North America to within a few thousand kilometers of South America's west coast. That's a big surprise, because it puts North America on the opposite side of Gondwana from the position it occupied in earlier, tentative paleogeographic reconstructions.

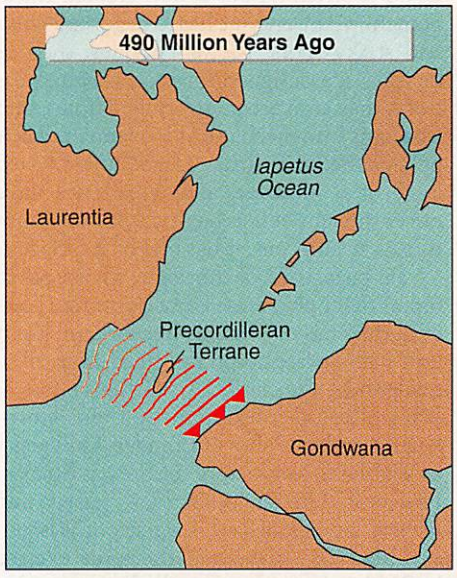
The revision may turn out to be important to researchers trying to understand "all these fantastic evolutionary things [that] were happening" at the time, says Dalziel, who first proposed the new geography. An evolutionary explosion was generating many of the life forms we know today, and the arrangement of drifting continents would have shaped such critical environmental factors as climate and sea level. The Argentine connection also provides a reference point for paleomapmakers as they ponder how all the continents, including North America, eventually gathered into a single supercontinent, Pangea, which formed about 250 million years ago.

Dalziel says he began suspecting that North America might once have lurked off the west coast of South America in 1991, when Eldridge Moores of the University of California, Davis, proposed how the continents might have been arranged in an even earlier supercontinent, called Rodinia, 750 million years ago. Moores suggested on the basis of geological similarities that Australia and Antarctica abutted the west coast of the ancestral North American continent, Laurentia, which lay at the core of Rodinia.

Dalziel, who had been thinking along the same lines, recalls that he then wondered how North America could have made its way "from somewhere adjacent to Antarctica to the position within Pangea that we know it had to have had." Taking into account such clues to Laurentia's wanderings as traces of Earth's ancient magnetic field frozen into its rocks, Dalziel plotted a meandering half-billion-year journey. The route took Laurentia on an "end run" around the west coast of South America to its final position in Pangea, where it collided with northwest Africa, pushing up the Appalachians. In mid-trip, according to a refinement offered by Luis Dalla Salda and his colleagues at the National University of La Plata, Argentina, the present eastern side of Laurentia ran smack into the coast of South America, right where the Andes rose hundreds of millions of years later.

At the GSA meeting, three speakers offered partial support for Dalziel's scenario. They confirmed that about 490 million years ago Laurentia and South America had been, if not in contact, close enough to exchange an 800-kilometer-long chunk of plate. First, William Thomas of the University of Kentucky explained how in 1991 he had inferred from U.S. geology alone that about 540 million years ago, a block of Laurentian crust had split away from what is now the Gulf Coast, never to return. At the time, he says, he had no idea where it might have gone.

The answer came in the second talk. The scheduled speaker, Ricardo Astini of the National University of Córdoba, Argentina, could not attend, but Thomas and Robert Hatcher of the University of Tennessee, the



Gone south. During a close encounter between continents, a chunk of ancient North America migrated to South America.

third speaker, delivered his talk for him. For 30 years, they noted, paleontologists have known that a crustal block now locked in the Andes north of Mendoza in far western Argentina, called the Precordillera, contains distinctively North American fossils of 540 million years ago. But Astini helped pin down the North American connection by drawing attention to similarities between the rock types and the sequence of strata in the Precordillera and the southeastern United States. "They are identical," says Hatcher. "It's amazing."

At an October workshop in Argentina, other geologists agreed that the Precordillera of western Argentina broke away from the Gulf Coast about 500 million years ago, say Thomas and Hatcher. But younger fossils in the Precordillera imply that before it "docked" on the South American coast, the block of crust spent some time on its own, as an island. That suggests that Laurentia and South America "were not in contact," says Hatcher, "but they could easily have been within 1000 to 2000 kilometers of each other," given the timing of the transfer.

That Laurentia was near South America 500 million years ago rather than 4500 kilometers off Africa on the other side of Gondwana is gratifying, says Dalziel, but he hasn't given up on the idea that they collided. Direct contact would eliminate any uncertainty about Laurentia's location, after all. Perhaps, instead of breaking all connections with North America before colliding, the Precordillera could have remained attached to it by a long, submarine plateau, much like the one that now connects the Falkland Islands to South America. North and South America might not have embraced during this dance of continents, but perhaps they stole a kiss.

–Richard A. Kerr

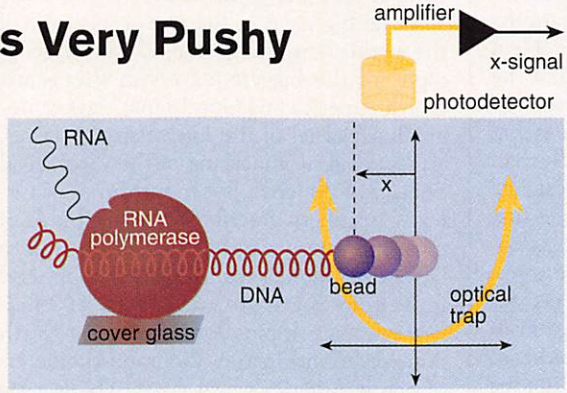
BIOCHEMISTRY

RNA Polymerase Gets Very Pushy

RNA polymerase doesn't appear to be an enzyme designed for hard work. Its main job is to slide along a DNA strand and splice free ribonucleotides into a messenger RNA chain based on the DNA template. But DNA is anything but a well-greased track: Somehow, the enzyme has to negotiate kinks and sticky spots. Motor enzymes, such as kinesin, are designed for such tough situations; they convert energy gained from other reactions into mechanical movement. RNA polymerase bears little resemblance to such molecules, however, and researchers have never thought of it as having a powerful motor. As it turns out, it has one of the biggest.

On page 1653 of this issue, investigators report that polymerase is not only more powerful than motor proteins like kinesin, but it uses fuel—pyrophosphates freed from ribonucleotides during RNA synthesis—just as efficiently. A consortium of three labs—headed by Jeff Gelles of Brandeis University in Waltham, Massachusetts; Steven Block of Princeton University in New Jersey; and Robert Landick of the University of Wisconsin, Madison—measured the force exerted by RNA polymerase as it pulled on a strand of DNA whose far end was caught in a laser-based trap called an "optical tweezers." The tug approached 14 piconewtons; other motor proteins pull at a strength of up to 6 piconewtons.

That combination of power and efficiency indicates why the enzyme can move along a DNA strand with apparent ease. Moreover, the technique gives scientists a whole new window on the interactions between the enzyme and the DNA substrate. Measuring force and displacement may allow them to analyze whether the enzyme moves one base at a time or in longer jumps, and how regulatory proteins such as transcription factors affect that movement. Other researchers are powerfully impressed. "It's one of the most elegant bits of biophysics that's been applied to transcription," says physical biochemist Peter von Hippel at the University of Oregon. "This adds a whole new dimension to understanding how the enzyme articulates with the [DNA] strand."



SOURCE: S. BLOCK

Transcription tug of war. As RNA polymerase transcribes a DNA strand, it pulls a bead attached to its far end from a low-energy spot in an optical trap formed by a laser beam. When the energy of the beam equals the force exerted by the enzyme, the polymerase stalls; that force is indicated by the bead's displacement *(x)*.

The major obstacles RNA polymerase encounters on a DNA strand are so-called "supercoiled" structures where the DNA helix is further twisted around itself. The polymerase has to overcome these constraints to keep a continuous hold on one strand. That would seem to require a lot of motor power, yet nobody had determined how much drive the polymerase actually had.

The team made this measurement by first fixing the polymerase to a glass cover slip while preserving its transcription activity. The enzyme, held stationary and unable to move along a DNA strand as it normally does, instead pulls the strand toward itself. Then the researchers fastened the far end of the DNA strand to a polystyrene bead just 0.5 micrometers in diameter. The bead is held under the "optical tweezers," an interferometer developed by Block and several colleagues which traps the bead at a low-energy spot at the center of a laser beam.

Then, says Gelles, "you start transcription by adding ribonucleotides." The polymerase starts tugging at the DNA strand, pulling the bead toward areas of more energy and higher resistance. The enzyme stalls when the resistance level of the beam matches the power of the enzyme's tug. A photodetector atop the apparatus measures the displacement of the bead, which is converted into a measurement of the enzyme's force.

Measuring "how much it takes to stall the enzyme is a completely new twist," says von Hippel. In addition to exhibiting the 14-piconewton force, RNA polymerase produces that force as efficiently as do "traditional" motor enzymes, converting about 10% to 20% of the free energy available from one cycle of ribonucleotide addition into mechanical energy. The polymerase, says Gelles, is the first member of what might be an unappreciated class of nucleic acid motor enzymes.

What excites Block about this work is the potential for getting a "blow-by-blow description of the polymerase in real time." Other experiments have indicated that RNA pauses and reverses at various points along the template, and may occasionally jump 10 bases at a time. Observing the changes in force exerted by the polymerase as it encounters different features of the substrate—individual DNA bases, or regulatory or suppressor proteins—should reveal which of those features are sending which signals. (For more on transcription and chromosomes, see the special section beginning on p. 1585.)

The present apparatus, however, can't measure much beyond overall stalling force. So the researchers are building a force-feedback clamp. Instead of maintaining a constant force on the bead, the clamp will change force to hold the bead steady as events at the polymerase end alter the tugs; researchers can then correlate the force changes with the transcription events. "This paper," says biophysicist Hermann Gaub of the University of Munich, Germany, "is opening up the field."

–Claire O'Brien

Claire O'Brien is a science writer in Cambridge, U.K.

MEETING BRIEFS

Plasma Physicists Seek New Uses for the Legacy of Fusion

LOUISVILLE, KENTUCKY—On 6–10 November, plasma physicists gathered here for their first major meeting since Congress decimated funding for fusion-energy research. Organizers noted optimistically that attendance had dropped only 10% from 1994. There was a more genuinely hopeful sign among the papers and posters, however. The 1285 physicists in attendance are eagerly applying hardware and concepts developed over decades of fusion research to new tasks—everything from spacecraft propulsion to understanding magnetic fields in the cosmos.

Sounding Out a New Dynamo

Magnetic fields seem to be the universe's ultimate weed, sprouting spontaneously in galaxies, stars, and planetary cores—wherever there is a conductive plasma or liquid metal. Just where they come from is a puzzle: It takes an organized, large-scale electric current to generate a field, and there's no obvious source of current in any of these objects. Dynamo theorists, who study how magnetic fields arise, thought they had one thing straight: Fluctuating electric fields—created by effects such as heat-driven convection, fluid buoyancy, or energetic plasma instabilities—ultimately drive the "cells" of current that add up to produce the large-scale magnetic field. But this picture just got a new twist.

New measurements presented at the conference by Hantao Ji, who recently moved from the University of Wisconsin to the Princeton Plasma Physics Laboratory, imply that those currents can be generated by something as simple as pressure fluctuations, analogous to sound waves in an ordinary gas. That proposal would amount to a major extension of dynamo theory, says Scott Robertson of the University of Colorado, Boulder: "You ask, 'Is it all driven by the same mechanism?' Ji [in his talk] was saying, 'No—it's not the same.' That's heavy stuff."

The new insight into how plasmas can generate magnetic fields comes from a child of the fusion program, a device called the reversed-field pinch (RFP). RFPs were originally thought up as a way to confine a hot plasma long enough for its ions to fuse, producing energy (*Science*, 14 July, p. 154), but they have also turned out to be a good laboratory system for studying how magnetic fields can spontaneously take root in a plasma. The basic setup is a donut-shaped container with current-carrying coils wrapped the short way around the donut. These coils create a "toroidal" magnetic field—a field looping the long way through the donut.

When plasma gets dumped into the container, however, this field spontaneously begins to twist away from the toroidal direction, at an angle that increases toward the container's walls. At the wall itself, the field actually reverses its direction, creating a toroidal component of the field oriented in the opposite direction from the field applied by the coils. This configuration gives the device its name—and also keeps fusion plasmas securely imprisoned.

The plasma's turbulent motion is the immediate cause of this new, spontaneous field, says Stewart Prager of the University of Wisconsin, a collaborator in the experiments. As each packet of conductive plasma moves through the existing magnetic field, it generates a tiny current. If these fluid elements simply sloshed back and forth, the currents would average to zero. But the kind of turbulence found in an RFP generates small-scale magnetic fluctuations as well as small-scale motions. The plasma's jitter and the magnetic fluctuations act in phase to generate a net, large-scale current, producing a new large-scale magnetic field—the dynamo field that creates the twist.

What shakes the plasma in the first place, according to dynamo theory, is a fluctuating electric field, generated in the RFP by fine-scale instabilities—in effect, tiny electrical storms driven by the plasma's internal energy. Working with RFPs at the University of Tokyo, the University of Wisconsin, and the Electrotechnical Laboratory in Tsukuba, Japan, Ji and co-workers set out to test this picture. They began by measuring the small-scale electric and magnetic fluctuations to see if they had the correct magnitudes and phases to produce the observed large-scale dynamo fields. But the observations "didn't match up," says Ji.

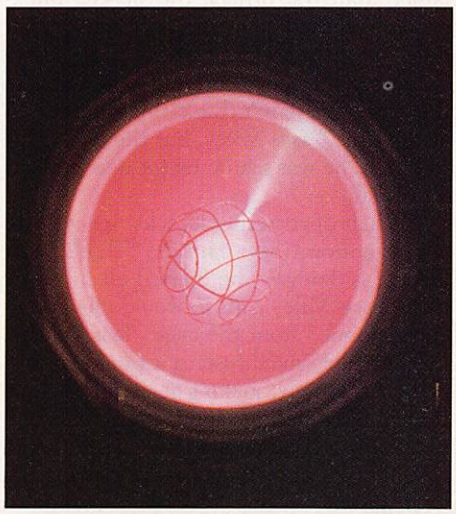
Ji speculated that the missing element might be pressure fluctuations—a phenomenon dynamo theorists have neglected, because so much of plasmas' behavior depends on electric and magnetic fields rather than on pressure. Ji realized, however, that sufficiently strong pressure fluctuations, possibly caused by new instabilities, could play the role of an electric field in knocking the fluid elements about. "This time it was a success," he says, as the probes showed that the pressure and magnetic fluctuations together could generate the dynamo field. Further measurements showed that the standard dynamo worked fine when the plasma was tenuous, but the new effect took over in denser plasmas. This dividing line is an observation that theorists have yet to explain.

Could the new dynamo have wider implications in a universe that is overgrown with magnetic fields? Katia Ferrière, a theorist who studies astrophysical dynamos at the Observatoire Midi-Pyrénées in Toulouse, France, points out that simple arguments about the strength of turbulence at different size scales suggest that such pressure fluctuations should be small in settings like the galaxy and the interior of the sun. Nevertheless, she says the new effect "is impossible to rule out" based on existing observations. Ji, for his part, is scouring the astrophysical literature for any hints that the effect could help explain why magnetic fields cling to the cosmos like kudzu.

Move Over, Wernher von Braun

In *The Evolution of Useful Things*, the engineer Henry Petroski marveled at "the almost limitless functions to which a single form can lead." Petroski was writing about the humble paper clip, but several presentations at the plasma conference show that the same may hold for much grander devices—reactors meant to confine a plasma while its ions fuse, generating energy. At least two such devices might find potential new uses in spacecraft propulsion.

Plasma-powered rockets may well turn out to be as far-fetched as fusion reactors have been accused of being. But while one scheme is admittedly visionary, aiming at fusion-powered spaceships, the other aims at a comparatively down-to-earth application: low-power thrusters, driven by electric cur-



YIBIN GU

Ball of fire. Ions jet from a 4-cm metal cage in what could be a prototype satellite thruster.

rent rather than fusion, for maneuvering satellites. Either way, researchers note that, in a time of sharp cutbacks in the fusion program, they have little to lose by proposing imaginative new uses for their technology. "My strategy is, we'll concentrate on [propulsion and] anything else I can think of now," says George Miley, director of the Fusion Studies Laboratory at the University of Illinois, Urbana-Champaign.

Working plasma thrusters, proposed for use on next-generation communications satellites, already exist, based on flat, electrically charged grids that draw beams of ions through a nozzle. These devices, however, tend to be short-lived, as the ions erode the fine mesh. But at the plasma physics meeting, Miley proposed that a fusion device invented by ITT's Philo Farnsworth—better known as the principal inventor of television—can provide a sturdier alternative. As modified by Farnsworth's co-worker Robert L. Hirsch, now president of Energy Technology Collaborative in Washington, D.C., the fusion device consists of a metal cage carrying a strong negative charge, which confines a low-pressure cloud of plasma that, Hirsch hoped, would serve as fusion fuel.

In this scheme, the symmetric negative charge would drive beams of positively charged ions into the cage's center, where they would collide and fuse. Unfortunately, says Hirsch, the fusion community has long favored schemes to use electric rather than magnetic fields to confine plasmas, and Department of Energy support for studies of the device's fusion potential has dwindled. But in the meantime, Miley found that even if the device has no chance of achieving fusion, it can serve as a thruster.

The cages for his fusion experiments were "all made by students—and it's a little bit tricky, because [the cages] have to be quite symmetrical," Miley explains. When a student wound a cage with one hole slightly larger than the others, the plasma unexpectedly started squirting only out of that hole—probably because of the near-perfect symmetry of electric potentials elsewhere in the cage. "We didn't think 'this is a thruster' at the time," says Miley, but the idea came to him as he was searching for new projects for his lab.

"When opportunity knocks, it's good to pay attention," says Hirsch. "That's what George is doing." The inertial electrostatic thruster, as Miley calls it, would rely on energy siphoned from the cage's bias voltage. Like other plasma thrusters, it would use propellant sparingly, making it useful for low-thrust, long-lifetime applications like maneuvering a communications satellite. And it should also last longer than existing flat ion thrusters, because the ions are channeled through an opening in the cage.

Terry Kammash, a professor of nuclear engineering at the University of Michigan, is plotting a more ambitious future for an erstwhile fusion machine. The device, called the magnetic mirror, actually functions more like a leaky bottle. It consists of current-carrying coils arranged in a cylinder, which generate a large bundle of magnetic-field lines inside the cylinder. At two points, stronger currents encircling the cylinder constrict the field lines, creating choke points that reflect ions following the field lines. The effect partly confines a hot plasma to the region between the constrictions, encouraging it to fuse. The mirror-fusion program "died on the vine" when it didn't live up to initial hopes, says Kammash.

But Kammash wants to make a virtue of the mirror machine's leakiness. Working at very high plasma densities, Kammash envisions making one end of a mirror leakier than the other and igniting a fusion reaction inside it. Plasma squirting out the leakier end would push the rocket. Kammash and his Michigan colleague Myoung-Jae Lee, who presented results in Louisville, calculate that such a machine, weighing about 500 metric tons and measuring less than 100 meters in length, could make a round-trip, crewed flight to Mars in a few months—compared to several years for a chemical rocket.

The fusion thruster "has characteristics that are quite nice as far as something you'd want to explore the solar system with," says William Emrich, a senior engineer at the National Aeronautics and Space Administration's Marshall Space Flight Center in Huntsville, Alabama. The rocket could be assembled in orbit, and its fuel weight would be "minuscule" compared to that of other rockets, Emrich says, adding, "You could go to Pluto with no problem."

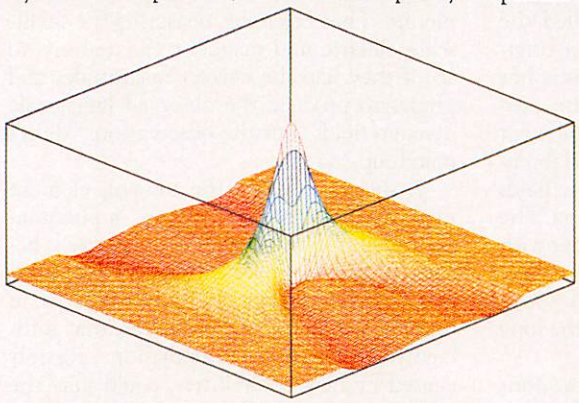
How to Detonate a Plasma

More than a decade ago, Princeton University plasma theorist Russel Kulsrud shook his head and summarized the problem this way: "I don't understand why things go bang."

He didn't mean familiar explosives like dynamite but plasmas, which can sit quietly for long periods and then detonate for no apparent reason. In the sun's corona, for example, huge arches of plasma rise above the sun's surface, apparently stable, until the structure suddenly erupts in the catastrophic events called coronal mass ejections. Likewise, in the donut-shaped fusion devices called tokamaks, a hot, confined plasma can suddenly "disrupt," hurling itself against the vessel's walls. Theoretical calculations generally show that if a quiescent plasma passes an instability threshold—say, as more material is poured into a cage of magnetic fields—the plasma should shiver and then rearrange itself to become stable again. But theorists simply may not have followed the plasma's contortions for long enough to see the explosion.

At the Louisville conference, Steve Cowley of the University of California, Los Angeles, a former student of Kulsrud's, presented theoretical work that takes that extra step and may go a long way toward explaining why things go bang. Working with a model of a plasma confined by magnetic field lines, which become "stretchy" when plasma gets loaded onto them, Cowley has found that a narrow, marginally unstable bundle of lines can undergo explosive contortions. Although the field lines try to snap back at first, Cowley found that, like overstretched elastic, they eventually lose their resiliency and give up the fight. The result is a "blast wave" effect that progressively destabilizes the rest of the plasma.

"What Steve has done is to find a concrete, three-dimensional, physical situation in which this phenomenon occurs," says Ravi Sudan of Cornell University. The calculation considers an instability that might develop in a magnetic arch on the sun, in which a thin bundle of field lines, loaded with relatively tenuous plasma, starts to bob upward through overlying, denser plasma. Because the endpoints of the field lines are fixed, as they are on the sun, the lines stretch like rubber bands. In the traditional picture they eventually resist the instability and snap back. But Cowley's nonlinear calculations show that as the field stretches even further, plasma expands in the deforming bundle, forming bulging, unstable "fingers" of instability. As the fingers thicken, the magnetic field weakens and loses its ability to snap back, resulting in explosive growth and launching a blast wave through the plasma.



STEVE COWLEY

Out of control. A fingerlike instability in a plasma begins its runaway growth in new theoretical calculations.

Cowley points out that similar instabilities develop in many explosively unstable plasmas. Although he cautions that the calculations must still be carefully compared to data from tokamaks and the sun, Cowley says, "this could be why things go bang."

–James Glanz

edited by CONSTANCE HOLDEN

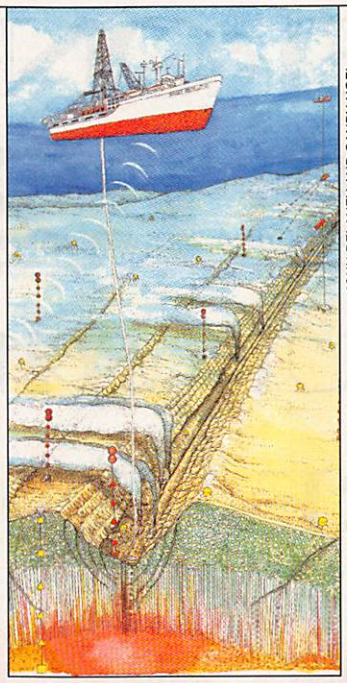
Undersea Observatory

A unique sea-floor observatory will soon be keeping a continuous watch on two undersea volcanoes off the coasts of Washington and Oregon, according to a plan announced last week.

The plan, which could cost $30 million, involves setting up instruments on the sea floor to monitor seismic activity, currents, temperature change, and marine biota at two volcanic sites for years at a time. "Nothing of this magnitude and duration has been done in the deep sea before," says Chuck Fisher, a marine biologist at Pennsylvania State University. Fisher is part of the team, led by scientists at the University of Washington and Scripps Institution of Oceanography, that developed the plan. Currently, says University of Washington oceanographer John Delaney, scientists are only able to study such underwater sites from the surface or in submersible flybys.

Part of the National Science Foundation's RIDGE (Ridge Inter-Disciplinary Global Experiments) program, the observatories, which will take 4 years to set up, will cost between $2 million and $3 million a year for at least 10 years, says Delaney. Some instruments are already deployed at the sites, which lie between 200 and 400 kilometers offshore along the Juan de Fuca ridge, where two of Earth's plates are moving apart. Volcanic eruptions and earthquakes are common in such areas, as magma from the mantle rises to fill the openings. The ridge is also lined with hydrothermal vents, where unusual microbial life forms thrive in superheated, mineral-charged seawater. "The many types of instruments in the observatories will be our time-space 'telescopes' into how volcanoes—in the presence of water—can sustain life without sunlight," says Delaney. Remote-controlled vehicles may eventually be docked at the sites to collect samples and data.

Richard Thomson, a physical oceanographer at the Institute of Ocean Sciences in Sidney, British Columbia, says the plan is "quite exciting, because it focuses people on one area for a significant period of time. We'll get an integrated look at the whole system for the first time."



JOHN DELANEY AND SANDY NOEL

Sea floor plan. Observatory includes seismometers; current meter moorings; cones where instruments can be inserted into Earth's crust; video cameras; positioning beacons; and transponders to measure sea-floor spreading.

AIDS Tumor Bank

AIDS researchers now have a new resource available to them: a bank of AIDS-related tumors with previously hard-to-obtain tissue and fluid samples from AIDS sufferers along with detailed clinical information.

The AIDS Malignancy Bank (AMB), which opened in October, has been a year in the making. Funded by the National Cancer Institute (NCI), it comprises repositories in five sites around the United States.

Cancer biologist Michael McGrath of the University of California, San Francisco, says that almost half of all AIDS patients get some type of malignancy. About 20% will get Kaposi's sarcoma, and 10% to 15% develop some form of non-Hodgkins lymphoma. Yet tumor samples from AIDS patients—especially samples with full clinical and demographic information—are often hard for researchers to get, says Ellen Feigal of NCI's cancer therapy evaluation program, who has been coordinating the creation of AMB. "There are very few people who can do important work on AIDS-associated lymphomas because there are only a handful of sites around the country that see those patients and get biopsy material," says McGrath.

Now the bank will be able to supply "all types of fluid, tissues, cells, and blood products" associated with AIDS tumors and see that every sample is accompanied by "a high-quality clinical pedigree" so researchers will know about patients' treatment and how they responded to it, says McGrath, who heads one of the repositories. He says the bank will also enhance the work of the newly established AIDS Malignancy Clinical Consortium, funded by NCI, which comprises 13 institutions doing "innovative" clinical trials.

Scientists wishing to obtain samples will submit proposals that will go through an independent review board and be as-

(continued on page 1573)

PETER SCHMIDT

Ironmaking the old-fashioned way. Hayas recreate ancient forge.

How Hayas Fired Iron

Recent research into ancient and modern ironmaking in Africa provides new evidence of innovation among early iron workers as they coped with some unusual properties of indigenous materials.

In the November-December issue of *American Scientist*, anthropologist Peter Schmidt of the University of Florida and National Park Service archaeologist S. Terry Childs combine ethnographic and archaeological observations with laboratory analyses to study ancient technology among the Haya of Western Tanzania.

Ironmaking is said to have originated in the Mediterranean region between 1600 and 1200 B.C., spreading to Africa around 600 B.C. Although the Haya stopped iron smelting at least 70 years ago, the researchers found elders who were able to recreate the technology with iron ore from an old mine.

The researchers compared slag and iron from the experimental furnaces to materials excavated from Early Iron Age (600 B.C. to A.D. 600) sites in Tanzania. They were surprised to find both the ancient and modern materials had an unusually high phosphorus content—apparently from the trees and grasses used as fuel. The phosphorus helped in the smelting process, but the impurity made the iron brittle and easily cracked when cold-worked into objects.

The Haya smiths were able to identify the different kinds of iron produced in smelting and found ways to deal with them. They reduced the brittleness by reheating the partially purified "bloom" of iron, steel, and slag. This released carbon, making the iron stronger and more workable. The smiths used iron that was too phosphorus-rich for toolmaking to make simple ritual items.

"The genius of the Haya iron workers in dealing with the difficult materials is much greater than we initially realized," says Schmidt. Chapurkha Kusimba, an archaeologist who studies African iron technology at the Field Museum in Chicago, agrees, saying the research reveals that "inventions were going on as a result of experimentation."

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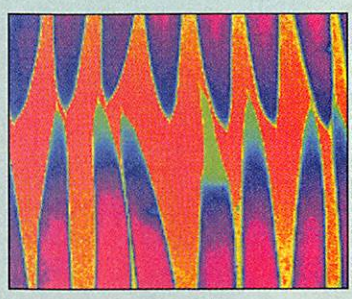
Pathology of a Tree

The Florida torreya, a conifer also called the stinking yew, is a cousin of the yew that produces the cancer-fighting substance taxol. It's also the rarest tree in North America. Although torreyas once flourished in ravines along northern Florida's Apalachicola River, they began to die in the 1950s, and the number has dwindled to a mere 1500. For years, people have proposed culprits for the tree's decline, such as the construction of a nearby dam, which may have warmed the ravine microclimate. Now scientists think they have found the true cause: a fungus that may have become deadly.



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What's eating yew? Needles imaged with fluorometry, which detects photosynthetic activity. Greenish-blue tips show damage from fungus.

Plant pathologist Gary Strobel of Montana State University, Cornell chemist Jon Clardy, and their colleagues collected samples from sick and healthy torreyas and analyzed their fungi. The presence of a fungus doesn't necessarily imply that it is harming a tree; most shrubs and trees contain fungi, says Strobel. But when the researchers took the most abundant of the 30 kinds of fungi found in the wild trees and applied it to cuts made on the limbs of greenhouse torreyas, the cuts soon turned brown and the needles yellowed. "We were able to cause some or most of the [symptoms found] in the field," says Strobel, whose team reports the work in the November issue of *Chemistry & Biology*. Grown in culture, the fungus, *Pestalotiopsis microspora*, produced three chemicals toxic to the torreya, two of which had never been previously identified.

Strobel thinks *P. microspora* may have lived amicably with the torreya until pollution or other stresses made the tree vulnerable to these toxins. Co-author Mark Schwartz, an ecologist at the University of California, Davis, hopes to find out whether treating the remaining torreyas with a fungicide can save them.

The tree's demise would be especially tragic, Strobel says, because the torreya, like its close relative the Pacific yew, may produce unique chemicals that could yield new drugs. Adds Keith Clay of Indiana University, who studies endophytic fungi, "This seems to be a really good case of how extinction could lead to the loss of potentially valuable research."

(continued from page 1571)

signed priority scores. Information about the bank can be accessed on the World Wide Web at **http://wwwicic.nci.nih.gov/amb/amb.html**.

EPA Crafts a Strategy For Science

Outside scientists have long urged the Environmental Protection Agency (EPA) to shore up the science behind its regulatory decisions. The agency has been responding—by adding more peer review and boosting extramural grants, for example—and now Robert Huggett, director of EPA's Office of Research and Development (ORD), has drafted a strategic plan that sets research priorities for the agency.

The plan, released 2 weeks ago, calls for EPA to focus its research on "the greatest risks to people and the environment." It identifies six near-term research foci: disinfection of drinking water, chemicals that interfere with endocrine systems, ecosystem protection, models for human health and exposure hazards, particulates harmful to health, and pollution prevention. At the same time, the plan calls for the agency to place less emphasis on routine data collection and low-risk hazards like municipal solid waste, says Joseph Alexander, ORD's deputy science director. EPA will also do more to integrate ecological and human health research.

"I think it's a big step forward. They've matured to ... understanding that there's a necessity to do some basic research," says ecologist Richard Fisher of Texas A & M University. Howard University toxicologist Bailus Walker, who serves on a National Research Council (NRC) panel that reviews EPA's research, agrees, adding that the plan "represents a good synthesis of the advice that has been coming to that agency from all directions" (*Science*, 31 March, p. 1903). EPA is seeking comments from its Science Advisory Board and the NRC panel on the draft, to be finalized next year. Meanwhile, Alexander says the draft plan will be a "pivotal tool" in coping with EPA's 1996 science and technology budget, which Congress wants to cut 10% compared to 1995 levels.

Eating Like a Greek

For years, physicians have been extolling the "Mediterranean diet"—heavy on fruits, vegetables, cereals, and legumes and light on animal fats—as a way to avoid cardiovascular disease. Now there's evidence from a study of elderly people in rural Greece, who've been on Mediterranean diets all their lives, that these eating habits can also prolong life.

The elderly provide "a living history of past nutrition," says Dimitrios Trichopoulos, an epidemiologist at Harvard School of Public Health and senior author of the study, which appeared in last week's *British Medical Journal*. Elderly Greek villagers "stick to the old customs, as elderly people usually do. ... That allowed us to evaluate, for the first time, the effect of a total diet" on survival rates. Previous studies on diet and longevity have focused on specific nutrients, Trichopoulos explains.

The study tracked 182 Greek villagers over age 70 for 6 years. According to questionnaires examining current eating habits, 57% of them followed traditional, healthy patterns in five or more of the eight food categories measured. Nontraditional eaters—who followed healthy patterns in two or fewer categories, made up 19% of the group. During the study period 53 people died. But those who followed tradition in six or more categories—for example, by eating large amounts of beans or bread, or by using olive oil instead of saturated fats—were only half as likely to die as those who adhered in three or fewer categories. No specific food group showed any relation to survival, but, says Trichopoulos, "taken together, they work." He says the villagers' regime—including whole-grain bread, beans, yogurt, feta cheese, vegetables cooked in olive oil, and moderate amounts of wine—helps explain the "Mediterranean paradox": the relatively long life-spans of people in this region despite their high total fat intake and high rates of smoking.

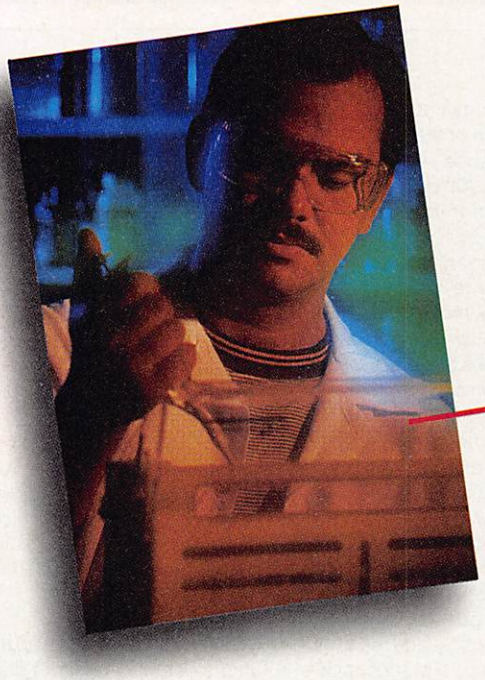
The study has "a very important message: A pattern of eating that includes all of these factors seems to be the healthiest," says R. Curtis Ellison, an epidemiologist at Boston University. But Dean Ornish, director of the Preventive Medicine Research Institute in Sausalito, California, and an outspoken advocate of a low-fat diet, notes the study doesn't necessarily mean the rest of us should imitate all aspects of traditional Greek dining. "People survived longer despite the olive oil and red wine," he says—"not because of them."

Science Standards: The Final Version

The final version of the National Science Education Standards has, after nearly 5 years of preparation and input from a cast of thousands, become available. The National Academy of Sciences released it on 6 December. The National Research Council has enlisted teams of educators around the country to be available to counsel locals about implementing the standards—a draft version of which (*Science*, 9 December 1994, p. 1637) is already in widespread use—in kindergarten through grade 12 education. The document is available for $23.95 (including shipping), plus 50 cents for each additional copy. Call 1-800-624-6242 or 202-334-3313.

Ron Lirette, Ph.D.

- Presently directing research activities and product development for Sigma BioSciences.
- Published in *Journal of Cellular Biochemistry, Journal of Virology, Journal of General Virology,* and *EMBO.*
- Presented abstracts at American Society for Cell Biology Conferences, Symposium of the Protein Society, Annual Meeting of The American Society for Virology, and International Symposium on Column Liquid Chromatography.
- Prior to Sigma, worked five years in a molecular biology group in the pharmaceutical industry.
- Also worked in academia at the Wistar Institute of Anatomy and Biology and at the University of Medicine and Dentistry of New Jersey.
- Received his Ph.D. from Louisiana State University.



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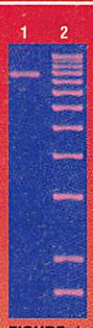

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FIGURE. Lane 1: 8.9 kb full-length RT-PCR product of APC transcript from 500 ng of total HeLa RNA isolated using TRIzol Reagent; lane 2: 1 Kb DNA Ladder.

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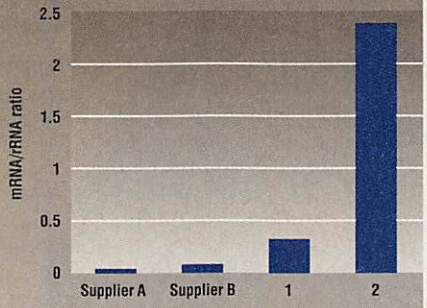

FIGURE. Comparison of mRNA quality purified by three different methods. Amount of GAPDH mRNA was compared to amount of rRNA remaining in each preparation. Supplier A: mRNA isolated by a guanidine isothiocyanate method and single-selection with oligo(dT) cellulose. Supplier B: mRNA isolated by a proteinase K-SDS method and single-selection with oligo(dT) cellulose. 1 and 2: mRNA isolated with the MessageMaker System and single or double selection with oligo(dT) cellulose.

Product	Cat. No.	Size
MessageMaker mRNA Isolation System	10551-018	10 reactions

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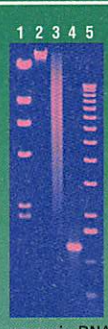

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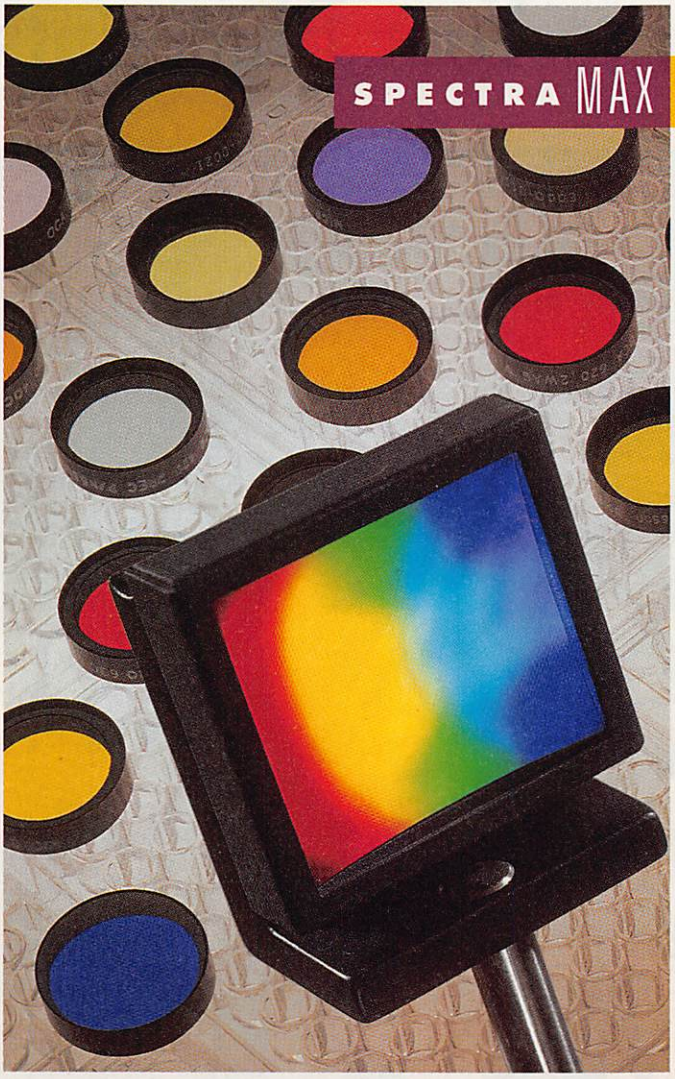

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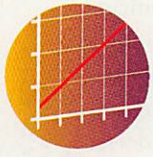
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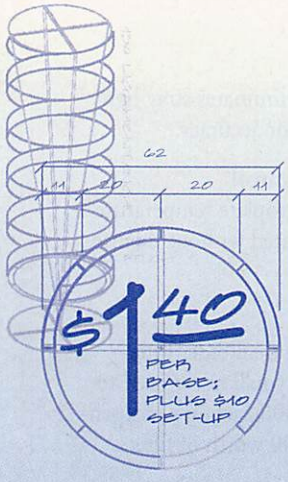

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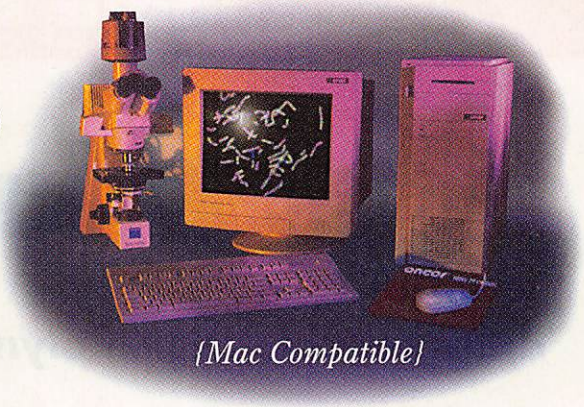

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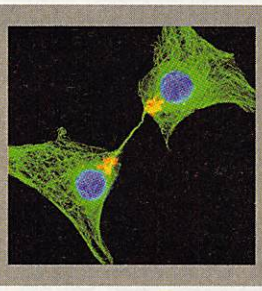

Immunofluorescence of NIH 3T3 fibroblast cell: Microtubules (green) are visualized with antitubulin-FITC antibody. Golgi apparatus (yellow) is stained with an anti-Golgi antibody conjugated to rhodamine. Nuclei (blue) are counterstained with DAPI. By Philip Huie, Stanford University, using the Mac-based Oncor Imaging System.

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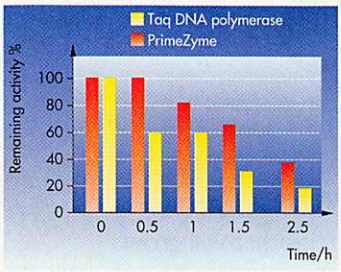

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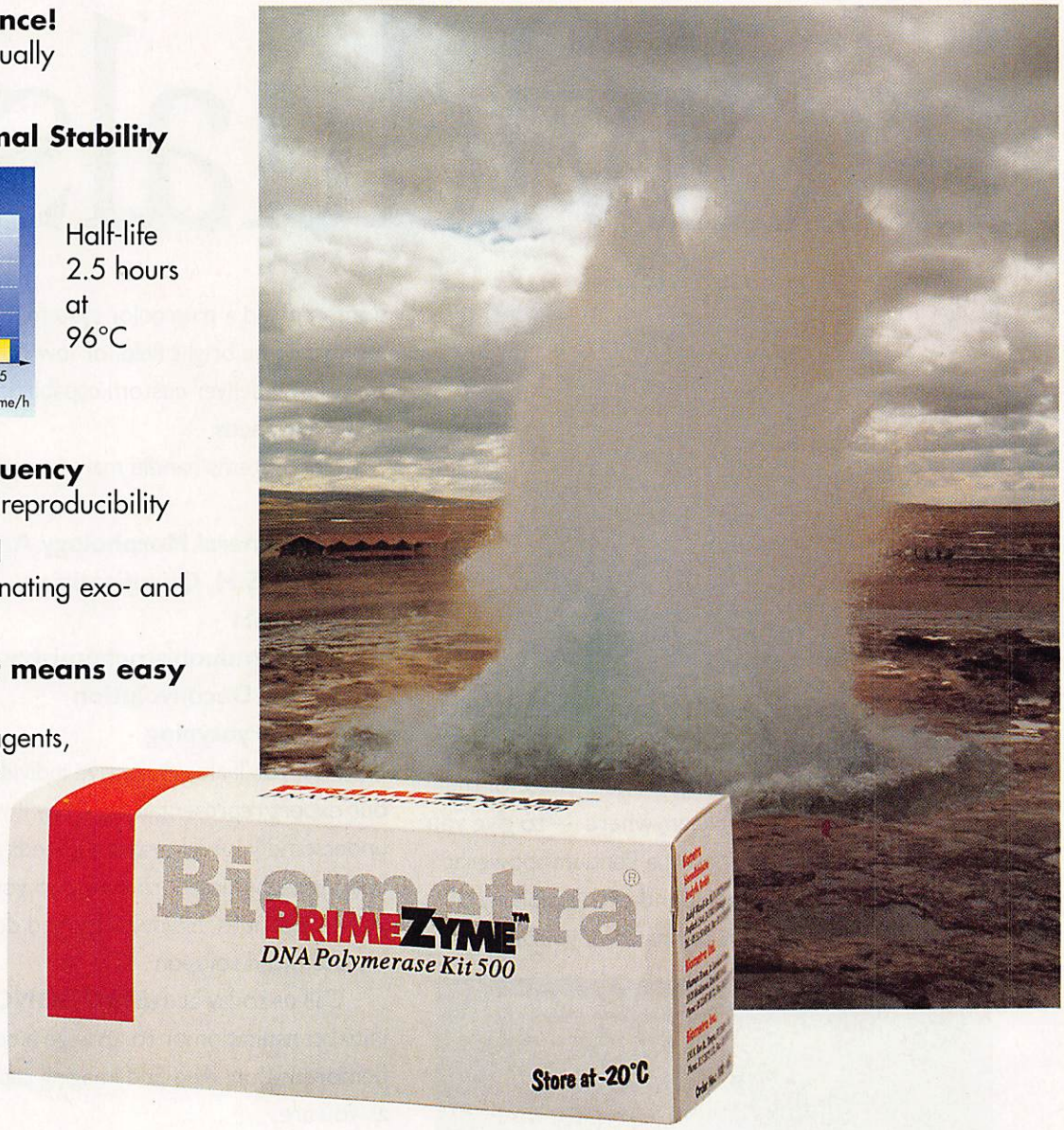

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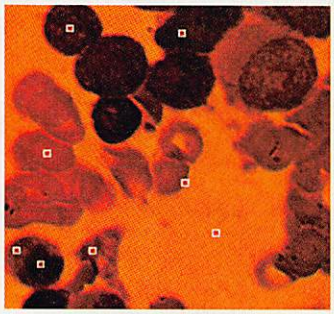

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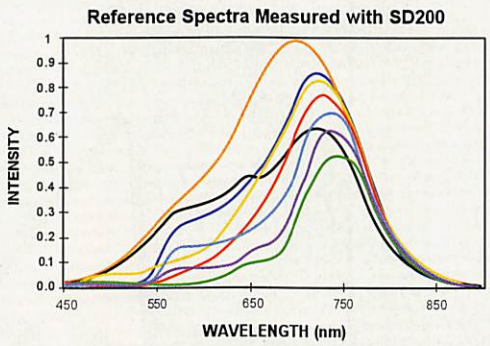

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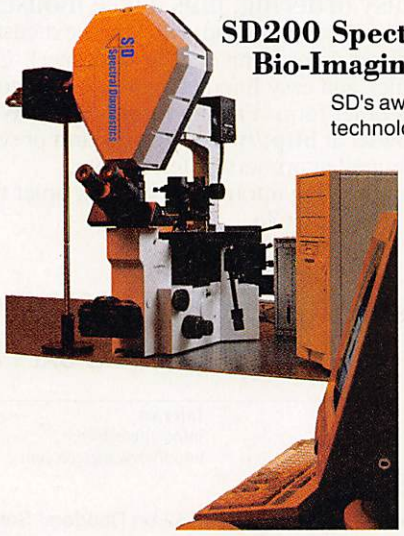

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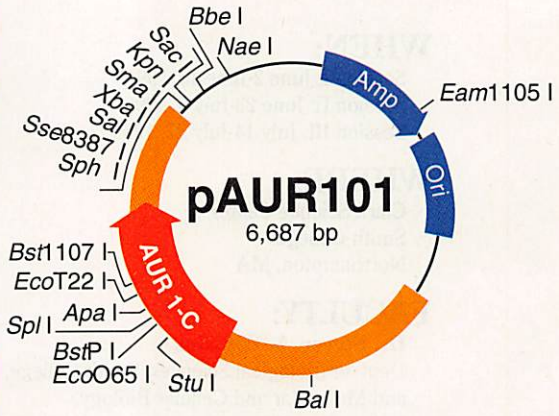

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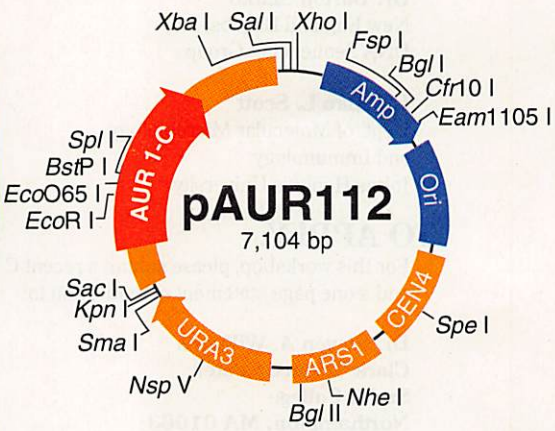

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How DNA Replication Originates

Researchers are at long last getting a look at the cellular machinery that ensures that DNA copies itself once—and only once—per cell division cycle

As the repositories of the genetic material, the chromosomes have long fascinated biologists—but rarely as much as they do today. Researchers are making progress on a series of broad fronts, leading to a better understanding of problems ranging from how chromosomes move during meiosis to how their structure influences gene activity. Many of these developments are discussed in the News reports beginning on this page and in the five Articles that follow, as well as in selected Reports.

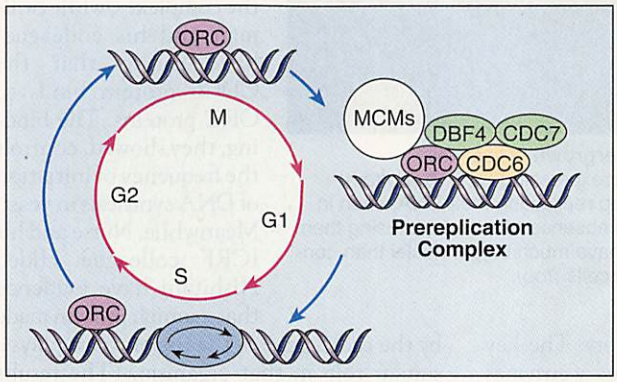
It's an essential prelude to cell division, and it sounds easy in the textbooks: Before a cell divides it has to replicate—or copy—its DNA. But concealed within that simple statement is a logistical nightmare, especially for the more complicated nucleated cells of higher organisms. Not only do they have to copy millions, or even billions, of base pairs of DNA just once, with exquisite accuracy and at just the right time in the cycle of cell division, but the DNA to be copied is distributed over numerous chromosomes—46 of them in the case of humans. And making matters even worse, the copying starts at hundreds to thousands of sites, some of which are triggered early in the synthesis (S) phase of the cell cycle, while others are triggered late.

Until recently, researchers had no idea how cells could complete this task without leaving some segments uncopied or making extra copies of others. But a flurry of recent work has finally begun to crack the puzzle. "It's exciting times in the field at the moment. [The work] is going to give us great insights into how replication is controlled," says molecular biologist Bruce Stillman of Cold Spring Harbor Laboratory, whose lab is among those focusing on DNA replication.

The answer, developed so far largely from studies in yeasts, lies in the interaction between short, specific stretches of DNA scattered throughout the chromosomes and a complex of proteins that binds to these "origins of replication," turning DNA replication on and off at the right moments. Researchers have also started to trace how these proteins are themselves controlled by the proteins that form the machinery of the cell cycle, the highly choreographed series of events that drives the cell to divide.

Understanding of DNA replication in more complex, multicelled organisms is less far advanced. But despite some problems identifying origins of replication comparable to those of yeast in these species, early evidence—including that in three papers in this special issue on the chromosome (pp. 1667, 1671, and 1674)—suggests that these organisms have replication control proteins similar to the yeast proteins. The research "is going very interesting places at the moment," says Julian Blow of the Imperial Cancer Research Fund (ICRF) laboratory in South Mimms, U.K., who studies DNA replication in the frog *Xenopus laevis*. "It looks like the basic processes in all eukaryotes will be the same."

Figuring out how DNA replication is controlled is an important challenge because accurate control is needed to maintain the integrity of the genetic material. Too little DNA synthesis, and essential genes might be lost, but too much could be equally bad. If the DNA is replicated more than once, cells may end up with extra copies of whole or partial chromosomes. Such abnormalities are thought to contribute to cancer development, possibly because they generate extra copies of cancer-causing oncogenes.



Getting ready. An essential step in initiating DNA replication seems to be the building on ORC of a "prereplication complex" containing several proteins, possibly including CDC6, the MCM proteins, the kinase CDC7, and its partner DBF4.

Yeast leads the way

Researchers have known for decades that DNA replication in bacteria and animal viruses is initiated when specific proteins bind to short sequences scattered through their genomes—that is, to the origins of replication. By leading to unwinding of the double helical DNA, a process aided by a helicase enzyme, this interaction eventually exposes the DNA to the polymerase enzyme that copies it. But researchers didn't get their first clue about whether a similar mechanism operates in higher organisms until about 15 years ago. That's when Ronald Davis's team at Stanford University and that of John Carbon at the University of California (UC), Santa Barbara, discovered "autonomously replicating sequences" (ARSs) in the genome of the budding yeast *Saccharomyces cerevisiae*. These are sequences that, when inserted into small circular pieces of DNA called plasmids, allow those plasmids to replicate in synchrony with yeast cell chromosomes.

In 1987, teams led by Walt Fangman and Bonnie Brewer of the University of Washington, Seattle, and by Joel Huberman of Roswell Park Cancer Institute in Buffalo, New York, confirmed that the ARSs are the counterparts of the bacterial and viral origin sequences. Since then work by several teams, including Stillman's and that of Carol Newlon at New Jersey Medical School in Newark, has further defined the nature of yeast origins. They consist of an 11 base-pair sequence found in all ARSs, plus two to three additional short sequences that are also needed for efficient initiation of DNA synthesis, all grouped within a 100 to 200 base-pair-long DNA sequence.

Once researchers had found that yeast has well-defined origin sequences, they were able to move quickly to find the proteins that interact with those sequences and presumably tell the DNA-synthesizing enzymes where and when to begin working. In 1992, Stillman and his then-postdoc, Stephen Bell, identified a core group of six proteins, collectively called the origin recognition complex (ORC), that seemed to fit the bill. Subsequently, the Stillman team, and those of Jasper Rine at UC Berkeley, Kim Nasmyth at the I. M. P. Research Institute of Molecular Pathology in Vienna, Austria, and Ira Hershkowitz at UC San Francisco, cloned the genes encoding the proteins, thereby opening the way to genetic dissection of their functions. The researchers found, for example, that mutations in ARS sequences that abolish their ability to initiate DNA replication also abolish ORC binding. And conversely, mutations in ORC

protein genes prevent initiation at ARS sites. Indeed, says Rine, "the ORC complex fulfills all the criteria for a replication initiator."

It soon became apparent, however, that ORC couldn't be acting on its own. In all normal cells, DNA replication occurs only during a specific phase of the cell cycle, called "S" for synthesis. But John Diffley and his colleagues at the ICRF South Mimms laboratory found that ORC remains bound to yeast origins all through the cell cycle. That means something else has to be acting with ORC to restrict initiation of DNA replication to S phase. "It's clear that ORC has an essential role in initiating replication ... but it's not sufficient," says Diffley.

Further evidence that ORC doesn't act alone came from experiments by the ICRF team indicating that one or more additional proteins bind to yeast origins at or near ORC late in the mitosis (cell division) stage of the cell cycle. They remain there until S phase begins and then are apparently lost until mitosis occurs again. Based on these findings, Diffley proposed that the first step toward DNA synthesis is the formation of what he calls the "prereplication complex," consisting of ORC plus the additional protein or proteins.

H. NISHITANI AND P. NURSE

Overgrown. Yeast cells overexpressing CDC18 *(bottom)* keep replicating their DNA, even in the absence of mitosis, causing them to have much bigger nuclei than control cells *(top)*.

The exact identities of these protein partners aren't known yet, but what researchers have already learned about them is helping them figure out how replication initiation ties into the cell cycle machinery. The key cogs in this machinery are a series of enzymes known as the cyclin dependent kinases (Cdks) because they require additional proteins, the cyclins, to do their job. Now researchers are finding that cells may achieve such tight control of DNA synthesis by having some of those same enzymes also regulate DNA replication.

In the fission yeast *Schizosaccharomyces pombe*, for example, the kinase produced by the *cdc2* gene and its cyclin partner is needed to push cells into mitosis, after which the cyclin is destroyed, inactivating the Cdk. But Paul Nurse of ICRF's London lab and his colleagues have discovered that the kinase has another role: While it's active it prevents replication from being initiated a second time. "That explains why there is only one S phase per cell cycle," says Nurse. "Only when [the mitotic Cdk] is destroyed do you lose the inhibiting signal."

And the Cdks apparently direct their inhibitory signals at components of the prereplicative complex. In a collaborative effort, the Nasymth and Diffley teams have found that the comparable Cdks of budding yeasts block formation of the complex. What's more, these researchers have added an intriguing twist to the story. They've found that these very same kinases are needed to trigger DNA replication during the S phase.

Having the same enzyme first activate DNA replication and then inhibit re-formation of the prereplication complex once it has triggered one round of DNA copying is an extremely efficient arrangement, Nasmyth says: "It's built into the logic of the cell cycle that you can't trigger the prereplication complex until you have gone through mitosis."

The targets of the kinases have not yet been identified, but possible candidates include ORC itself and another protein that combines with ORC in prereplication complexes. This is the product of the *cdc6* gene of the budding yeast *S. cerevisiae*. Diffley's group has genetic evidence that Cdc6 is needed for formation of the complexes, while Stillman and his colleagues have shown that the CDC6 protein binds to ORC proteins. The binding, they showed, controls the frequency of initiation of DNA synthesis in yeast. Meanwhile, Nurse and his ICRF colleague Hideo Nishitani have evidence that a similar protein made by the *cdc18* gene of the fission yeast plays a similar role in that organism. (The results appear in the 3 November issue of *Cell*.) "CDC18 not only initiates, but its high level is sufficient to drive cells into S without other protein synthesis," Nurse says. CDC18 (or CDC6) could be a target of the mitotic kinases, he says, but for now "that's pure speculation."

What about mammals?

The identities of the targets of the cell cycle kinases is only one of a great many questions that still need to be answered about how initiation of DNA replication is controlled. Among other things, for example, researchers want to know how the helicase and DNA polymerase enzymes get plugged into the system. But perhaps the biggest question of all is whether the results researchers are obtaining in yeast will also apply to mammals and other multicelled creatures. The main reason for doubt is that researchers have so far failed to pin down specific DNA sequences as origins of replication comparable to the ones in bacteria, viruses, and the yeast.

Some studies suggest, for example, that DNA synthesis is initiated anywhere over long sequence stretches. Others have come up with much shorter specific sequences, more like those in yeast. "Everyone was confused because all the measurements seemed valid. Yet they were leading to contradictory conclusions," says Roswell Park's Huberman.

Still, researchers are beginning to make progress in resolving the contradictions. One reason for the confusion may be that initiation "depends on the context," not just on specific sequences, as Marcel Méchali at the Institut Jacques Monod in Paris puts it. In the 10 November issue of *Science*, Olivier Hyrien of the École Normale Supérieure in Paris, Méchali, and Chrystelle Maric, also of the Institut Jacques Monod, report that in early *Xenopus* embryos, a time when embryonic genes are not transcribed, DNA replication initiates randomly at sites spread throughout the ribosomal rRNA genes. But later in development, when those genes are being transcribed, initiation sites are restricted to the spaces between the genes. This change from broad to narrower initiation zones may be due to alterations in the structure of the chromatin, the complex of proteins and DNA that form the chromosomes.

What's more, the apparent split between those who find broad initiation zones and those who find short ones may not be as severe as it initially seemed. Recent research suggests that broad initiation zones actually contain a small number of "hot spots," where initiation is more likely to occur than in other parts of the region. The differentiated cells of higher eukaryotes may have specific origins, although they appear to be more spread out than those of yeast.

But there's an even stronger indication that the yeast work may apply to the higher eukaryotes: It's beginning to look as if the rest of their replication machinery is similar to that of yeast, even if their origins aren't. The similarity starts with ORC itself.

In one of the three papers in this issue, the Stillman team describes the cloning of genes from the human and from the plant *Arabidopsis thaliana*, the nematode worm *Caenorhabditis elegans*, and fission yeast whose sequences suggest that they are those species' versions of the *ORC1* and *ORC2* genes of budding yeast. In the second, Mike Botchan's team at UC Berkeley reports the cloning of the *ORC2* and *ORC5* genes of the fruit fly *Drosophila melanogaster*.

Not only do the structures of these genes suggest that ORC proteins have been conserved in evolution, but in the third paper, Botchan's team, in collaboration with Rine's,

reports that the fruit fly's ORC2 gene, when put into mutant yeast, can replace some of the functions of yeast ORC2. "Since the complex is so conserved, we think this means that the basic mechanism of [origin site] recognition is conserved," Botchan says.

And the same may be said for the proteins that combine with ORC. One clue comes from a phenomenon called "licensing," discovered in the late 1980s by ICRF's Blow and Ronald Laskey of the University of Cambridge, U.K. Their studies of DNA replication in extracts of *Xenopus* eggs indicated that initiation of DNA replication requires some factor that can only gain access to the chromosomes during mitosis, a time when the nuclear membrane has broken down. Then, once replication has taken place, this licensing factor, as they called it, is somehow lost or inactivated. Once the nuclear membrane has regenerated, it can't reach the chromosomes again until another round of mitosis begins. This behavior, Blow and Laskey proposed, would ensure that DNA could not replicate more than once in any given cycle.

Now some of the molecules needed for licensing are turning out to belong to a family of proteins, known as MCM proteins, that was originally discovered in yeast but is now known to be present in all eukaryotes. This spring, the Blow and Laskey teams, and also that of Haruhiko Takisawa of Osaka University in Japan, showed, among other things, that antibodies to the *Xenopus* MCM3 protein remove licensing-factor activity from *Xenopus* egg extracts, while adding back the MCM proteins restores licensing activity.

The behavior of the MCM proteins in *Xenopus* resembles that of the proteins that combine with yeast ORC to form the prereplication complex. And because MCM proteins are found in yeast, the supposition is that they may in fact be part of that complex. Indeed, there may be another resemblance as well. Blow's group has found an as-yet-unidentified material in *Xenopus* that's needed for replication initiation, in addition to the MCM proteins. "We speculate that it's going to be a frog CDC6," he says, referring to the protein needed for prereplication complex assembly in yeast.

Taken together, the work in yeast and the other eukaryotes is building a picture of a complex initiation machinery centered on ORC. As Stillman describes it, "The concept has emerged that ORC is a landing pad for a whole bunch of other proteins" that come and go at precise times during the cell cycle to regulate DNA replication. And even though researchers still have a way to go to work out the precise identities and functions of all those proteins, they have also come a long way. "Two years ago we had no idea that all these proteins were involved," Stillman says. "Now at least we know what to focus on."

–Jean Marx

ARCHITECTURAL PROTEINS

Protein Sculptors That Help Turn On Genes

Inside a living cell lies perhaps the only place on Earth where a serious accounting problem is solved by something resembling art. In mammals, more than 100,000 genes must blink on or off along the chromosomes at precisely the right times, at just the right intensity, for the body to develop and function normally. But the protein switches that bind to DNA and turn genes on are in such short supply that if each of these proteins, known as transcription factors, had to act by itself, the cell would have far too few of them to give precise control of so many genes. And that's where the cell's artistic talents come into play.

Molecular biologists have known for about a decade that, rather than deploying the gene-regulating proteins one at a time, the cell dispatches groups of them in various combinations to turn on particular genes, thereby greatly expanding the number of distinct switches that can be formed. More recent work now suggests that cells go one step further, using "architectural proteins" to sculpt many of these protein clusters into precise three-dimensional shapes. The architectural proteins, which are themselves part of the transcription factor complexes, usually do this by binding to the DNA and bending it, often sharply, thereby bringing together other members of the complex, which are bound to separate segments of a gene's control region.

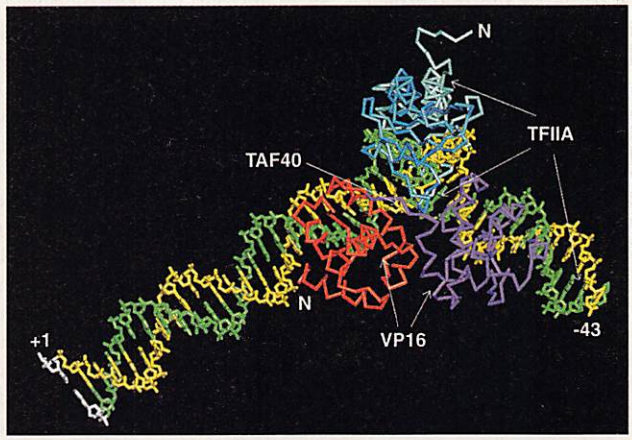
Without this sculptural talent, the transcription factor clusters would not function normally. "The three-dimensional organization of these complexes is critical for controlling the expression, and level of expression, of various genes," notes Stephen Burley of Rockefeller University. As a result, architectural proteins are turning out to play a key role in physiological processes ranging from inflammation to sex development.

The first indications of the existence of architectural proteins came in the early 1980s when Donald Crothers of Yale University discovered that certain proteins from the bacterium *Escherichia coli* could bend DNA, in one case wrenching 90-degree kinks in the molecule. The significance of that bending wasn't understood at the time, but in the course of his research, Crothers pioneered a method of inferring the existence and location of bends in DNA. The method sifts out bent DNA molecules from straight ones by exploiting the fact that in gel electrophoresis, bent DNA moves more slowly than does straight DNA of the same size.

Crothers's method was soon put to use by Howard Nash and his colleagues at the National Institute of Mental Health in Bethesda, Maryland, who were the first to identify a function for an architectural protein. When certain viruses infect *E. coli*, they insert their DNA into the bacterial genome. At the time, in the mid-1980s, a bacterial protein called integration host factor (IHF) was known to be needed for the insertion, although no one knew exactly what it did. Nash was trying to find out.

After ruling out the possibility that IHF was an enzyme, Nash recalls, he began investigating a structural role for the molecule. He applied Crothers's method and found that IHF bends DNA. He also noted that IHF binds to DNA's minor groove, the small space between its helical twists, an unusual trait for a DNA-binding protein. Nash's subsequent work, together with that of Arthur Landy's group at Brown University, showed that as a result of the bending, two other proteins, bound to separate segments of the bacterial DNA, were able to meet. This interaction, the researchers found, was necessary for viral DNA insertion.

But the DNA bending induced by IHF soon proved to have a much broader role in normal bacterial functioning. In 1990, Sydney Kustu's team at the University of California, Berkeley, noted that the protein



NIKOLOV *ET AL.*, *NATURE* **377**, 119–128

Around the bend. DNA bound to TBP *(blue)* and TFIIB *(red, magenta)* is L-shaped. The arrows indicate where other transcription factors in the complex bind.

strongly stimulates the transcription of a cluster of genes involved in nitrogen metabolism in *Klebsiella pneumoniae*, a close relative of *E. coli*. What's more, they showed that it bends the DNA at a site located between two other proteins needed for the first step in gene activity, the copying of the genes into RNA: the enzyme RNA polymerase, which does the copying, and the nitrogen fixation regulatory protein, NIFA. As a result of the bending, the two proteins moved together. That meeting, Kustu proposed in a landmark paper in *Cell*, triggered transcription. Kustu's model set a precedent, says gene transcription expert Tom Maniatis of Harvard University: "It was the first example of a DNA-bending mechanism required for gene activation." But it wasn't the last.

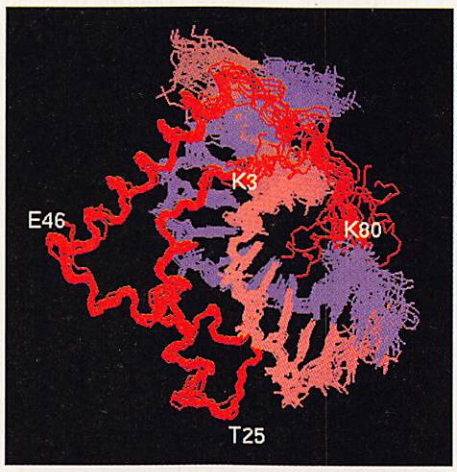
Two years later, a group led by Rudolf Grosschedl of the University of California, San Francisco (UCSF), found evidence that DNA bending plays a role in eukaryotic gene transcription. While studying a protein called LEF-1 (for lymphoid enhancer-binding factor 1), which regulates a gene crucial for the differentiation of key immune cells called T cells, Grosschedl and his colleagues noticed that it shares a peculiar property with IHF: binding to DNA's minor groove. Using Crothers's method, the UCSF team found that LEF-1, like IHF, bends DNA sharply.

Those similarities prompted the researchers to see whether LEF-1 might perform a function similar to that of IHF in assembling protein complexes, Grosschedl says. And they found indirect evidence that it does. They showed that the protein, which they obtained from the mouse, could substitute for IHF in promoting integration of viral DNA into the *E. coli* genome, an action that depends on DNA bending.

Since then, Grosschedl's group has obtained biochemical evidence that DNA bending is also important for LEF-1's normal function as a transcription factor. Those results indicated that LEF-1 plays matchmaker for two critical transcription factors by bending the DNA between them. And they noted that only when the match was made would transcription proceed at a normal pace.

But the most direct evidence for LEF-1's architectural role came just a few months ago. In the 31 August issue of *Nature*, Peter Wright and his co-workers at Scripps Research Institute in La Jolla, California, who worked in collaboration with Grosschedl's group, published an atomic-scale structure of LEF-1 interacting with DNA. The structure, obtained by nuclear magnetic resonance (NMR) methods, reveals that LEF-1 contacts DNA's minor groove, shoves a side chain of one its amino acids between a DNA base pair, and bends the DNA helix a spectacular 120 degrees.

That picture had in fact been forecast a few months earlier when a very similar DNA-bending portrait was found for another protein that, structurally speaking, looks very much like LEF-1. The protein, called SRY (for sex-determining region, Y chromosome), turns on the genes necessary for the formation of the testis and other male sex organs in mammals (*Science*, 29 September, p. 1824). This past June, Marius Clore, Angela Gronenborn, and their colleagues at



LOVE *ET AL.*, *NATURE* **376**, 791–795

Twisted. Binding of the transcription factor LEF-1 *(red)* puts a major kink in DNA.

the National Institutes of Health (NIH) determined the NMR structure of SRY bound to DNA, showing that the protein bows the DNA about 80 degrees. Clore and his colleagues believe the SRY-induced bend snaps together distant proteins into a compact complex that triggers transcription. However, the other components of that complex have not been identified.

While SRY and LEF-1 buttress protein complexes that regulate specific genes, another architectural protein, the transcription factor known as the TATA-binding protein (TBP), acts on many different genes. TBP typically binds to the so-called "TATA box," a short sequence of alternating thymidine (T) and adenosine (A) nucleotides found at the start of most genes. This protein serves as a generic scaffolding that can support different molecular sculptures depending on the gene. "It's like a hook on a wall. You can hang different pictures from it," explains structural biologist Milton Werner, who works in Clore and Gronenborn's lab at NIH.

About 50 proteins fold into each TBP complex, so determining the interactions of all of them is a daunting task. Nevertheless, researchers got their first inkling of how it might form when they determined that TBP bends DNA. That happened in 1993 when two groups—Peter Sigler's at Yale and Burley's at Rockefeller—simultaneously published x-ray crystallography structures of TBP clinging to DNA. The papers revealed "an amazing bend" of 70 degrees in the DNA, says Burley.

In more recent work, Burley's team has gone one step further by showing how at least one part of the transcription machinery fits into the bend. In the September 14 issue of *Nature*, Burley and his colleagues published the crystal structure of DNA bound both to TBP and TFIIB (for transcription factor IIB), a cog in the general transcription machine that includes RNA polymerase. That structure shows an L-shaped fragment of DNA created when TBP clutches the L's outside corner; the TFIIB nestles in the nook of the L. In this case, the bending creates a unique structure that TFIIB recognizes and can hang on to. "It's a beautiful story," comments Werner. "It indicates the structural context of how other proteins assemble on a scaffold."

And the architectural protein story doesn't stop there. At least eight other proteins are accepted members of the group, although their mechanisms of action are less well understood than those of LEF-1, SRY, and TBP. One of these is HMGI(Y), which is required to turn on genes needed for inflammatory responses, such as those encoding cell adhesion molecules that enable white blood cells to stick to, and then slip through, blood vessel walls and into injured tissue.

Recent biochemical work by Maniatis's group suggests that HMGI(Y) binding distorts the DNA, although this bending is much less than that produced by the other architectural proteins. Because HMGI(Y) seems to interact directly with other proteins in the complex, Maniatis thinks this architectural protein functions primarily as a clasp that helps transcription factors bind to DNA and links those factors to each other, rather than using its DNA-bending powers to bring transcription factors together. But until scientists determine the crystal structure of HMGI(Y) interacting with the double helix, the anatomy of this complex will remain unresolved.

Indeed, many questions remain about architectural proteins and their mechanisms of action. One big mystery, for example, is how transcription complexes like the one organized by LEF-1 activate RNA polymerase at the gene they regulate. Amazingly, some of these complexes form thousands of base pairs away from the site where the polymerase binds. In the case of LEF-1, the gap is about 4500 base pairs.

Also unclear is how many architectural proteins exist, although researchers suspect the total may soar into the hundreds. "It remains to be seen how many genes are dependent on architectural proteins," says Grosschedl. "But more and more examples are coming to light." Still, even the examples that have seen the light so far have been enough to illuminate the artistic genius of the cell.

–Ingrid Wickelgren

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CHROMATIN STRUCTURE

Molecular Machines May Aid Gene Expression

Each human cell contains a full 2 meters of DNA. To solve what could be the ultimate storage problem, the DNA is wound like thread around a spool of proteins known as histones, forming the complexes known as nucleosomes. These, in turn, make up the chromosomes that fit easily in the cell nucleus. Some geneticists speculate, however, that such compulsively neat storage raises problems of its own: Like clothes in an overpacked closet, the DNA is hard to get at. Now those researchers are glimpsing how the cell exposes the genes to the enzymes and other building materials needed to transcribe them into RNA.

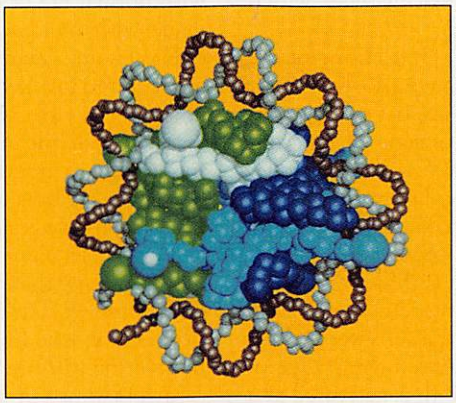
In the last 18 months, chemists, molecular biologists, and geneticists have unveiled a battery of molecular machines, energy-consuming proteins in the cell's nucleus, that disrupt the histones. Apparently working in conjunction with transcription factors—DNA binding proteins that trip switches called regulatory elements to turn genes on and off—these protein complexes remodel the nucleosomes, allowing the transcription machinery access to the DNA.

The discovery is pumping new life into the old idea that histones regulate genes by selectively exposing them to transcription. That theory took brief hold in the 1970s after researchers studying the structure of chromatin, the DNA-protein mixture that makes up the chromosomes, discovered the thread-and-spool structure of the nucleosomes. It fell into disrepute, however, as evidence grew that genes are regulated mainly by transcription factors. Now it seems that histones may have a part to play in gene regulation after all.

Histone disruption "may supply an additional layer to [gene] regulation that overlays the control exerted by transcription factors," says molecular biologist Peter Becker of the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany. A second line of research, meanwhile, is showing how some histones can have a targeted effect on the activity of genes, suppressing the activity of some and not others in a phenomenon called gene silencing (see box). Still, researchers warn that many of the histone results are preliminary, and some are controversial. "The field is in flux. There are new results every month, and the models change every month," says Becker.

There had, in fact, been hints all along that chromatin structure might be important for gene activity. Arrays of nucleosomes roughly similar to those in the living nucleus can be reconstructed in the test tube. Genes in such nucleosome templates are transcribed into RNA far more slowly than genes in naked DNA. Many other experiments have shown, conversely, that when genes are activated in yeast, fruit fly, and mammalian nuclei, the structure of the nucleosomes containing their regulatory sequences changes. But it remained unclear whether such disruption is needed for gene activation to occur, or is simply its consequence.

An inkling that nucleosome disruption may be a separate step that gets genes ready for transcription came in a surprise finding last year. While studying activation of heat shock genes, a team led by molecular biologist Carl Wu of the National Cancer Institute (NCI) in Bethesda, Maryland, found



ARENTS AND MOUDRIANAKIS, *PNAS* **90**, 10489–10493 (1993).

Heart of the matter. DNA in the nucleus is wound around histone cores.

that adding GAGA, the transcription factor that helps activate the genes, to nucleosome preparations containing GAGA's regulatory sequence changed the structure of the nucleosomes. Previous work had led the researchers to expect that, Wu says. What was surprising was that the transcription factor alone couldn't modify nucleosome structure. "It required ATP absolutely," he says. There's no evidence that GAGA itself consumes energy, so the result, which appeared in the 10 February 1994 issue of *Nature*, implied that, besides transcription factors, "you needed a specialized energy-consuming machine to destabilize the nucleosome," says Wu.

A few months later, a team led by genetic biochemist Craig Peterson of the University of Massachusetts Medical Center in Worcester and molecular biologist Jerry Workman of Pennsylvania State University in University Park identified a candidate for the molecular machine: A humungous protein complex called SWI/SNF (pronounced "switch/sniff"). Yeast geneticists had suspected for several years that SWI/SNF—which contains at least 11 different SNF proteins and SWI proteins, one of which can break down ATP—plays a role in histone-gene interactions. They'd found, for example, that disabling any one of six of the protein subunits blocks the activity of large numbers of yeast genes, and that the effect is reversed by disabling the genes encoding histones. One interpretation of the "double mutant" experiments was that SWI/SNF releases certain genes from the histone's repressive influences.

In more recent work, reported in the 1 July 1994 issue of *Science*, Peterson and Workman provided direct evidence for that idea. They found that when they added purified histones to test tubes containing the transcription factor GAL4 and its target regulatory element, GAL4 binding to the element dropped to one hundredth of its normal level. Adding SWI/SNF and ATP to the histone-containing preparations, however, brought GAL4 binding up again, by 30-fold. "It looked like SWI/SNF might be loading the transcription factor onto the nucleosomes," says Peterson.

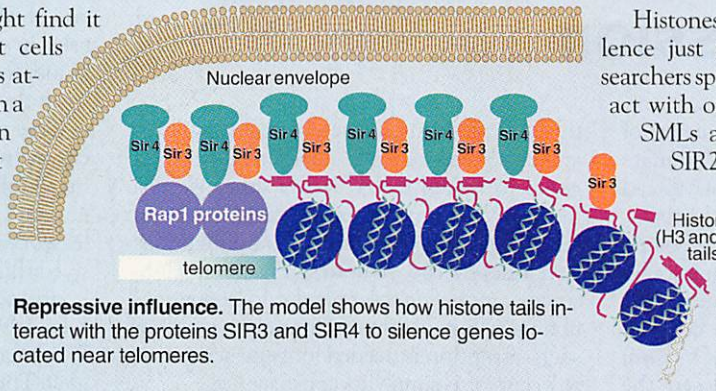
But some researchers suspect that SWI/SNF's apparent nucleosome remodeling activity is a test-tube artifact. "What raises the red flag," says biochemist James Kadonaga of the University of California, San Diego, "is that they have to add so much" SWI/SNF to restore binding. What's more, he says, it's not clear that the mononucleosome assay used "is a good model for chromatin."

Those concerns don't worry Peterson, however. "You'll never prove [SWI/SNF's role] without a shadow of a doubt. But, hey, some people say HIV doesn't cause AIDS," he says. What's more, Peterson now has evidence from living cells that confirms the importance of SWI/SNF. In yet-to-be-published work, Peterson has found that blocking the production of the SWI/SNF subunit SWI1 in modified yeast cells prevents GAL4 from binding to its regulatory elements. "The role in vivo is very similar to the role in vitro," says Peterson, although the cells in the experiment were modified to ensure that GAL4 regulatory sites were embedded in nucleosomes, which is not usually the case.

The SWI/SNF complex may be only the first of several molecular machines that disrupt chromatin. NCI's Wu has isolated from the fruit fly another protein that upsets the structure of nucleosomes in an energy-dependent manner. Wu has since purified the protein, which he calls NURF, for nucleosome remodeling factor, but says he can't discuss its characteristics because they will be reported in an upcoming issue of the journal *Cell*. EMBL's Becker has still another candidate for an energy-consuming nucleosome disrupter—a protein he named CHRAC for

Histones Hush Yeast Mating Genes

Although a mere human might find it difficult to fathom why, yeast cells sometimes find other yeast cells attractive. Yeasts usually indulge in a rather tame form of reproduction called vegetative budding, but every so often some yeast cells convert to the "a" type, and others to the "α" type. These a and α cells find each other alluring and fuse, in the yeast version of sex.



Repressive influence. The model shows how histone tails interact with the proteins SIR3 and SIR4 to silence genes located near telomeres.

Now studies of how yeasts prepare for sex are helping resolve an issue that has plagued histone researchers. For decades, these researchers have sought evidence that histones, which are in intimate contact with DNA throughout the genome, can regulate specific genes (see main text). And the study of yeast sex has shown that in one situation they can. Molecular geneticist Michael Grunstein and his colleagues at the University of California, Los Angeles, have direct evidence that histones help regulate the genes that trigger the two mating types, which are located in the silent mating loci (SML).

For good measure, a team led by Daniel Gottschling of the University of Chicago and Grunstein's team have also shown that histones have a specific effect on the telomeres, the structures at the chromosome tips that suppress activity of genes in the near vicinity. (Also see article by Zakian on p. 1601.) Taken together, "it's the most remarkable data," says molecular biologist Gary Felsenfeld of the National Institutes of Health in Bethesda, Maryland.

The current findings grew out of work Grunstein performed a few years ago. He noticed that yeast cells with certain mutations in the amino-acid tails of two histone proteins, designated H3 and H4, have trouble mating, although the cells were otherwise normal. Grunstein's team soon pinpointed the source of the yeast impotence: Normally, only one SML is activated at a time, but in the mutant cells both were active. As a result, the yeast cells fail to produce pheromones and so lose their attraction for one another. Gottschling, Grunstein, and their colleagues found that those same histone mutations abolish the repressive effect of yeast telomeres on nearby genes, without affecting other genes.

Histones don't have the specificity to silence just these particular genes. So researchers speculated that histone tails interact with one or more proteins specific to SMLs and telomeres—perhaps RAP1, SIR2, SIR3, and SIR4, proteins already known to be required for silencing SMLs and genes near telomeres. Results reported in the 24 February issue of *Cell* by the Grunstein team have confirmed that suspicion.

To tag normal and mutant H3 and H4, researchers genetically engineered hybrid proteins in which the enzyme glutathione S-transferase (GST) was fused to the histone tails. After mixing each hybrid one at a time with each of the RAP and SIR proteins, the researchers then used glutathione-coated beads that bind GST to precipitate the hybrid histones, which carried with them the proteins they bound.

The result was clear-cut, says Grunstein. Hybrids constructed with normal H3 or H4 tails bound SIR3 and SIR4. But hybrids made with mutant tails did not. The experiment "brings the chemistry and genetics together. The regions [of H3 and H4] that are required for silencing in vivo are the regions that bind SIR3 and SIR4 in vitro," he says.

And while the initial experiments were done in the test tube, in the past few months, Grunstein and his postdoc Andreas Hecht have found evidence that histones and SIR proteins behave in the same way in live yeast. Hecht found that he could precipitate from cell extracts a clump of proteins including SIR3, SIR4, H3, and H4, indicating that in living cells the four proteins are bound in a large complex. The evidence suggests that "histones are involved in silencing [the SML and the telomeres], and they do that by interacting with the SIR proteins," says Grunstein.

And Grunstein suspects that the histones' sphere of influence doesn't end with yeast SML and telomeres. "It's a leap," he says, "but perhaps histones interact with other proteins in other parts of the genome" to regulate other genes. That remains to be seen. But for now, histone enthusiasts have at least one firm example of how their favorite proteins can have specific gene control functions.

–R.N.

chromatin accessibility activity.

The Becker team stumbled across CHRAC this summer while investigating whether any DNA binding protein other than transcription factors could gain access to DNA in the nucleosomes. The answer proved to be yes. The researchers found that even DNA-cutting restriction enzymes, which come from bacteria, organisms that have no nucleosomes, remodel nucleosome templates. And, once again, ATP was required. Like transcription factors, "restriction enzymes don't normally require energy," says Becker. So using "brute-force biochemistry," he and his colleagues went in search of a putative energy-consuming cofactor for the enzymes, isolating CHRAC, which appears capable of remodeling nucleosomes, suggesting that it too helps expose genes for transcription.

And no one thinks that will be the end of the list. Indeed, at the Chromatin and Transcription meeting held from 24 to 29 June in Snowmass, Colorado, Workman's postdoc Thomas Owens-Hughes announced that he had isolated a protein from human cells that consumes energy and disrupts nucleosomes when GAL4 fastens to its binding site. They call this protein NDF for nucleosome disruption factor. "The present conclusion is that there is not one chromatin remodeling activity, but various activities," Becker says.

But despite the wealth of new information, there are still some big unknowns, says molecular biologist Richard Young of the Whitehead Institute in Boston. "How," he asks, "is [SWI/SNF] being brought to the regulatory sequences where they supposedly do their remodeling?" What's more, the most controversial idea, that the molecular machines fine-tune the activity of individual genes by acting on histones and nucleosome structure, is unproven. "Even for genes where SWI/SNF is clearly required for absolute expression, there's no evidence that it is also part of normal regulatory control," says geneticist Fred Winston of Harvard Medical School.

That notwithstanding, biochemist Roger Kornberg of Stanford University in Palo Alto, California, takes an optimistic view. "The remodeling of chromatin for transcription is a far bigger story than we know at the moment," he says. "It's going to be the life's work for the next generation."

–Rachel Nowak

The Centromere: Hub of Chromosomal Activities

A. F. Pluta, A. M. Mackay, A. M. Ainsztein, I. G. Goldberg, W. C. Earnshaw*

Centromeres are the structures that direct eukaryotic chromosome segregation in mitosis and meiosis. There are two major classes of centromeres. Point centromeres, found in the budding yeasts, are compact loci whose constituent proteins are now beginning to yield to biochemical analysis. Regional centromeres, best described in the fission yeast *Schizosaccharomyces pombe*, encompass many kilobases of DNA and are packaged into heterochromatin. Their associated proteins are as yet poorly understood. In addition to providing the site for microtubule attachment, centromeres also have an important role in checkpoint regulation during mitosis.

Centromeres have multiple roles during mitosis and may also have essential functions in interphase. (i) The centromere is the site of formation of the kinetochore, the buttonlike structure at the chromosomal surface that binds spindle microtubules and regulates chromosome movements in mitosis. The DNA sequence that specifies centromere location on the chromosome is referred to as the *CEN* locus. (ii) The centromere is the final locus of sister chromatid pairing in mitosis and, therefore, must receive the signal that triggers the release of sister chromatids at the metaphase-anaphase transition. Centromeres presumably contain the machinery necessary to effect this separation. (iii) The centromere is involved in cell cycle checkpoint control. In most cells, centromeres of mitotic chromosomes that have not yet achieved a stable bipolar orientation on the spindle send a signal that delays the onset of the metaphase-anaphase transition. (iv) The centromere acts as a marshaling area for the chromosomal passenger proteins, proteins that transfer from the chromosomes to the mitotic spindle during metaphase or anaphase (*1*). These are thought to be mitosis-specific cytoskeletal proteins (Fig. 1).

In this review, we discuss several aspects of centromere structure and function that have received particular attention in recent years. For other reviews we refer the reader to references (*2–4*).

The Two Classes of Centromeres: Point and Regional

Although centromere functions are conserved in all cells, centromeres exhibit bewildering structural variability between species. This ranges from holocentric chromosomes, where microtubules attach along the entire length of the chromatid, to chromosomes with localized centromeres, where microtubules attach to a single region that often appears constricted relative to the chromosome arms. Although holocentric chromosomes occur in many arthropods and plants as well as in the well-studied genetic model organism *Caenorhabditis elegans* (*5*), their structure remains poorly understood. This review will therefore focus on localized centromeres.

There are two classes of localized centromeres, point and regional centromeres. Point centromeres have been identified in the budding yeasts *Saccharomyces cerevisiae* (*3–5*), *Schizosaccharomyces uvarum* (*6*), and *Kluyveromyces lactii* (*7*). These compact loci encompass three conserved DNA elements (CDEs): CDEI, CDEII, and CDEIII (*8*). [The point centromeres of *Yarrowia lipolytica* have a different sequence organization (*9*).] The 25–base pair (bp) CDEIII is absolutely required for centromere function and is the binding site for an essential protein complex, termed CBF3 (*10*). Mutations in CDEIII or in genes encoding the CBF3 polypeptides disrupt chromosome segregation (*3–5*). The 8-bp CDEI sequence also appears in the promoters of a number of genes. Its binding factor, Cpf1p (*11*), is a transcriptional activator when bound to these noncentromeric sequences. This is paradoxical because transcription directed toward the core *CEN* sequences abolishes centromere function in vivo (*12*). CDEI and CDEIII are separated by CDEII, an A:T-rich region whose primary sequence is not well conserved but whose length is important for centromere function.

The point centromere is packaged into a compact nuclease-resistant chromatin structure of ~250 bp (*13*) that binds a single microtubule (*14*). Several findings suggest a model in which this structure may be assembled around a modified nucleosome. A reduction in amounts of core histones H2B or H4 alters centromeric chromatin (*15*), and *CSE4*, a gene required for chromosome segregation, encodes a highly divergent histone H3–related molecule (*16*). The human centromeric polypeptide CENP-A is also a very divergent histone H3 (*17*). Because the preferred length for CDEII, 78 to 86 bp, corresponds to roughly one turn of the DNA around the nucleosomal core, this model predicts that CDEI and CDEIII might be juxtaposed in space (Fig. 2).

Regional centromeres, the other class of

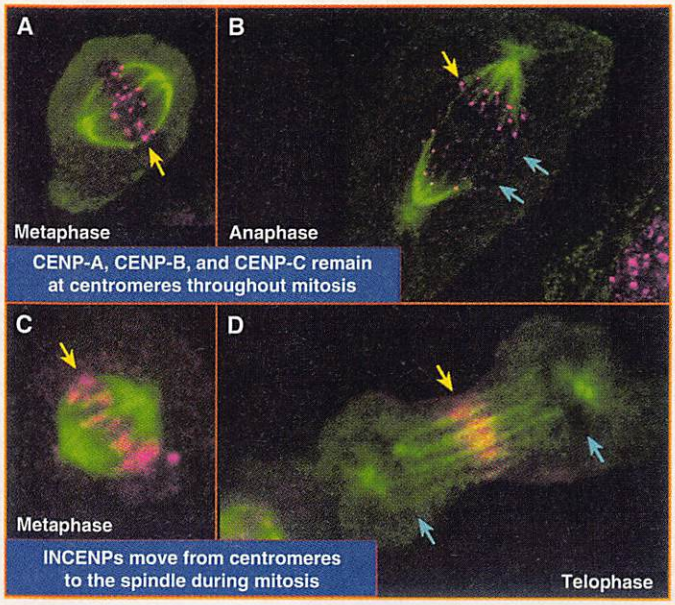


Fig. 1. Different behavior of intrinsic proteins of the centromere and chromosomal passenger proteins during mitosis. Both the CENP antigens [(**A**), labeled red with autoimmune serum] and passenger proteins, the INCENPs [(**B**), labeled red with rabbit serum], concentrate at centromeres during metaphase. The CENP antigens remain at centromeres during anaphase (**C**), whereas the INCENPs transfer to the overlapping microtubules of the central spindle (**D**). The position of the CENP and INCENP antigens in mitosis is indicated by yellow arrows. Microtubules are labeled green, and the blue arrows indicate the position of the chromosomes during anaphase and telophase.

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localized centromeres, encompass kilobases of DNA and include both unique and repeated DNA elements. The organization of the repeated DNA component can exhibit considerable polymorphisms between strains, as it does in *S. pombe* (*18*), or between individuals, as it does in humans (*19*). Whereas a single *CEN* locus accomplishes all aspects of centromere function in the point centromere, in regional centromeres different sequences may have become specialized to regulate kinetochore assembly and sister chromatid pairing.

Centromeres of *S. pombe* are the paradigm for the regional centromere. These loci span 40 to 100 kb (*20*), with the smallest functional centromeres containing ≥19 kb (*21*). The chromatin structure and functional organization of these centromeres are complex. The centromeres of *S. pombe* all contain a 4- to 7-kb central core sequence embedded within a domain containing both inverted and direct repeats. This central core contains several functionally redundant domains (*21*). A 2.1-kb region of the K/dg-type flanking repeat is also required for centromere function (*20*). This region confers a special chromatin structure on the central core sequences (*22*) and has a number of binding sites for proteins that have yet to be identified. It is not known if the bundles of two to four microtubules bound by *S. pombe* kinetochores (*23*) are associated with specific DNA sequences, as is the case in the point centromere, or if the regional centromere adopts a three-dimensional configuration that nucleates assembly of a proteinaceous kinetochore. It cannot be excluded that regional centromeres contain point *CEN* loci that have yet to be identified.

In most organisms, centromeres are composed of heterochromatin, a specialized form of chromatin that remains condensed throughout the cell cycle, replicates late in the S phase, and suppresses the expression of most genes. Like classic heterochromatin, *S. pombe* centromeres are not transcribed and can exert a position effect on reporter genes that are inserted into the centromeric array (*24*). Certain mutants that affect silencing of the mating type loci (*clr4*, *rik1*, and *swi6*) also affect centromeric silencing (*25*). These mutations also compromise the fidelity of chromosome segregation, suggesting that transcriptional suppression and centromere function are mechanistically linked. Recent work on the *swi6*$^+$ gene reveals that Swi6p is enriched in centromeric and telomeric heterochromatin (*26*). Swi6p has a sequence motif, the chromo domain, that has been found in several proteins that are involved in the stable repression of gene expression (*27*). One of these proteins, HP1, is concentrated in heterochromatin (*28*), particularly at centromeres in mammals (*29*). The other protein, polycomb, is thought to stabilize chromatin domains in a transcriptionally inactive state.

In a further parallel with heterochromatin, the establishment of centromeric activity in *S. pombe* is subject to functional variegation. When certain artificial chromosomes are introduced into *S. pombe* cultures, a substantial difference in chromosome stability is seen between different colonies (*30*). Thus, centromere function may require not only a minimal set of sequences, but the acquisition of specific epigenetic modifications by those sequences.

A recent study suggests a fundamental similarity of organization between the *S. pombe* and *Drosophila melanogaster* centromeres. *Drosophila* centromere function requires *Bora Bora*, a 220-kb "island" containing complex sequence DNA (*31*). For full centromere function, *Bora Bora* must be flanked on either the 5′ or 3′ side by another ~200 kb of simple sequence satellite DNA. It has been suggested that *Bora Bora* nucleates kinetochore formation, whereas the simple-sequence DNA regulates sister chromatid pairing.

Several groups are attempting to define functional *CEN* sequences in mammals, with the ultimate goal of constructing stable artificial chromosomes. This approach is hindered by the extraordinary variability of centromeric DNA sequences between species. Only one centromeric sequence element is known to be conserved between primates and rodents. This is the 17-bp binding site for CENP-B (*32*, *33*). A summary of the centromere components of mammals is presented in Fig. 3. The "CENP-B box" is found in human α-satellite DNA, *Mus musculus* minor satellite DNA, and in a minimal functional form (9 of 17 bp conserved) in the 79-bp centromeric satellite DNA of *Mus caroli* (*34*). In African green monkey α-satellite DNA the CENP-B box occurs at a frequency ≤0.1% of that found in human cells (*35*), raising doubts as to whether the binding of CENP-B to this sequence can be a major determinant of centromere structure and function. Such concerns were previously raised by the demonstration that CENP-B protein and CENP-B box DNA are both undetectable on the human Y chromosome (*36*).

Current evidence indicates that α-satellite DNA does have an important role in centromere function. When incorporated into ectopic locations on chromosome arms, transfected α-satellite DNA can perturb the segregation of sister chromatids (*37*). Whether this occurs because of the assembly of extra kinetochores or because of effects on sister chromatid separation remains to be determined. One approach to identifying human centromeric DNA involves the use of telomere-mediated fragmentation of chromosomes within the centromere. This approach has enabled the localization of the Y chromosome centromere to a region of ~70 kb within the α-satellite array (*38*). The most stable of these fragmented chromosomes also retain unique sequences from the p arm of the Y chromosome. The parallel to *S. pombe* and *Drosophila* regional centromeres is noteworthy.

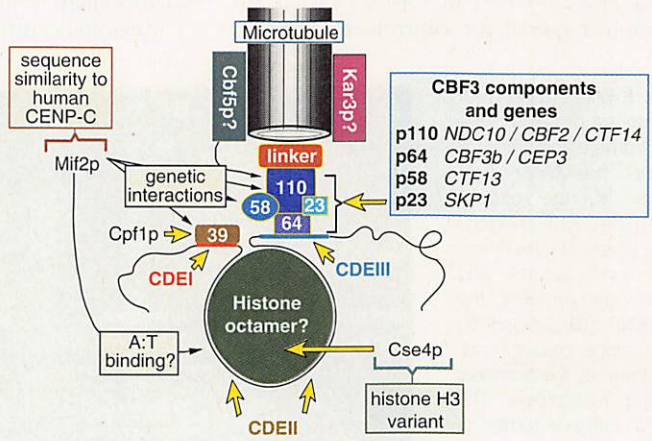

Fig. 2. Hypothetical model for the budding yeast kinetochore. The 125-bp *CEN* DNA is shown wrapped around a nucleosomal core, which may contain the histone H3 variant Cse4p (*16*). Whilst this model is consistent with the results of mutational analysis of the histones and length constraints on the CDEII sequence, direct evidence for such a configuration has yet to be obtained. Conserved DNA element I (CDEI, 8 bp) is complexed with the 39-kD Cpf1p [also known as CP1 and CBF1 (*11*)]. The four-subunit CBF3 complex binds the 25-bp element CDEIII. The CBF3 subunits include p110 [encoded by the *NDC10/CBF2/CTF14* gene (*68, 69*)], p64 [*CBF3b/CEP3* (*70*)], p58 [*CTF13* (*69*)], and p23 [*SKP1* (*71*)]. The detailed functions of these components are unknown, although p64 contains zinc finger motifs and has therefore been suggested to be the DNA binding subunit. p23 is conserved in species from *Arabadopsis* to mammals (*71*). The hypothetical linker molecule is inserted as a result of experiments demonstrating that CBF3 alone is not sufficient to cause *CEN* DNA to associate with microtubules in vitro (*72*). The kinesin-related protein Kar3p appears to be responsible for centromere movement on microtubules under in vitro conditions (*73*). Mif2p, which is required for chromosome segregation and spindle assembly (*74, 75*), interacts genetically with p39, p64, and p110. Cbf5p, which can bind to microtubules, also interacts genetically with p110 (*56*). Mif2p shares several short regions of amino acid similarity with the human kinetochore protein CENP-C (*75, 76*).

Centromeres May Function During Interphase

Whereas the functions of mitotic centromeres are well documented, the role of centromeres during interphase remains enigmatic. The persistence of condensed centromeres during interphase was first revealed by the staining of discrete foci in interphase nuclei with serum from autoimmune patients (*39*). These foci correspond to specialized heterochromatin domains (*40*). The demonstration that these foci of antibody staining colocalize with α-satellite DNA suggested a tight association of at least some "intrinsic" proteins with the centromere throughout the cell cycle (*41*). Association of these proteins with centromeres during interphase is apparently required for assembly of functional kinetochores. Microinjection of centromere antibodies during interphase disrupts kinetochore assembly and blocks progression through mitosis (*42, 43*). Thus, centromeres are not quiescent during interphase but require specific interactions with other proteins to prepare for their subsequent roles in chromosome segregation during mitosis.

Mammalian centromeres occupy distinct, nonrandom positions in the interphase nucleus. However, these centromeres are not entirely clustered or polarized as has been described in fission yeast (*44*) and *Drosophila* (*45*). Instead, mammalian centromeres tend to congregate near the nuclear periphery and around nucleoli, although they can also be found throughout the nuclear volume. These interphase centromere positions are not static, nor does their arrangement appear to conform to one pattern for all cell types. Rather, within a cell type, centromere distribution with regard to these two nuclear landmarks reproducibly changes during cell cycle progression (*46*) and in response to the functional (*47*) and transcriptional states of the cell (*48, 49*). This has led to the thinking that highly repeated sequences such as those at centromeres may organize the interphase nucleus in ways that influence gene expression (*50*).

The association of interphase centromeres with nucleoli in human cells is true not only for the acrocentric chromosomes in which centromeres and nucleolus organizer regions (NOR) are in relatively close proximity, but also for centromeres of non–NOR-bearing chromosomes (*49, 50*). Centromere proteins are detectable both ultrastructurally and biochemically in isolated human nucleoli (*51*). Furthermore, preliminary evidence has been obtained for a direct biochemical interaction between human kinetochore protein CENP-C (*52*) and the nucleolar transcription factor UBF (also known as NOR90) (*53*). Whereas the functional significance of these associations remains unclear, studies of human autoantibodies suggest that centromere associations with nucleoli may have important consequences. In particular, it has been postulated that a complex between nucleoli and centromeres is the dominant autoantigen in scleroderma spectrum disease (*54*).

A further link between centromeres and nucleoli involves Nap57p, a nucleolar protein from rat liver, which is thought to function as a chaperone for nucleolar proteins (*55*). Nap57p closely resembles Cbf5p, a *S. cerevisiae* protein of unknown function (*55, 56*) that interacts genetically with CBF3 (Fig. 2) and is also localized to the nucleolus (*56*). The detection of biochemical associations between centromeres and nucleolar proteins suggests that centromeres may have as yet undiscovered functions during interphase.

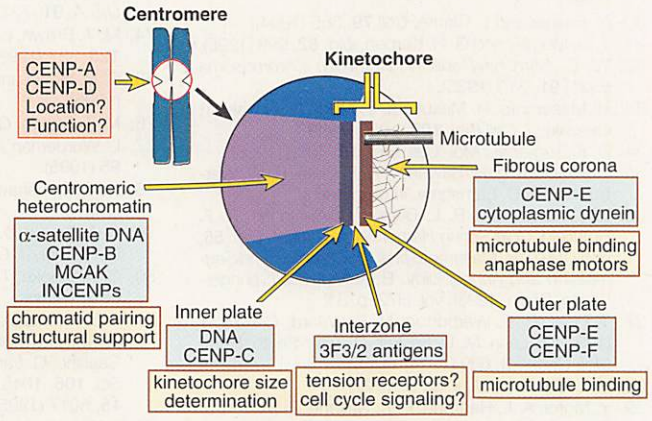

Fig. 3. Components of the mammalian centromere. The condensed centromeric heterochromatin, which may function as a structural foundation for the kinetochore, is rich in α-satellite DNA and its binding protein CENP-B (*33, 40*) but also contains the kinesin-related MCAK (*77*) and the INCENP chromosomal passengers (*78*). At the surface of the heterochromatin is the inner kinetochore plate. This structure contains both DNA (*79*) and CENP-C (*52*), with the latter being essential for kinetochore assembly (*43*). The outer kinetochore plate, which contains the kinesin-related CENP-E (*80*) and CENP-F (also termed mitosin) (*81*) is involved in microtubule binding. The interzone between the two plates contains the 3F3/2 antigens (*65*) and may be involved in tension sensing and cell cycle signaling. The fibrous corona contains microtubule motor proteins, both of the kinesin [CENP-E (*80*)] and cytoplasmic dynein (*82*) families. The detailed localizations of CENP-A [a divergent histone H3 that resembles yeast Cse4p but is not functionally interchangeable with it (*16, 17*)] and CENP-D [which appears to correspond to the RCC1 protein, a regulator of nuclear transport (*83*)] are not known.

Centromeres Signal the "Wait Anaphase" Checkpoint

When vertebrate cells enter mitosis, the nuclear envelope breaks down and chromosomes randomly encounter microtubules that emanate from opposing centrosomes. The ensuing processes of microtubule capture by kinetochores and the gradual establishment of a balance of forces between opposing kinetochores and centrosomes ultimately result in the establishment of the metaphase configuration with all chromosomes lined up at the midzone of a bipolar spindle and awaiting the onset of anaphase. If aneuploidy is to be avoided, it is crucial that the cell delay anaphase onset until all chromosomes have achieved this balanced alignment. In recent years, significant advances have been made in the characterization of this checkpoint.

In 1970, Zirkle proposed that a signal emanating from the chromosomes or spindle could prolong metaphase until both kinetochores of every chromosome had been attached to microtubules and brought to the metaphase plate (*57*). Dietz (*58*) had originally proposed and Nicklas (*59*) demonstrated that meiotic kinetochores could somehow sense tension. This led to the proposal that in the absence of tension produced by a stable bipolar attachment, kinetochores transmit an inhibitory signal that delays the metaphase-anaphase transition (*60*). Results consistent with this view have been obtained in *S. cerevisiae*, where cells with mutant centromeres exhibit a mitotic delay (*61*), and in humans, where microinjection of antibodies to centromeres also causes a mitotic delay (*42*).

A recent study provides direct evidence for the involvement of kinetochore tension in releasing cells from the "wait anaphase" checkpoint. In mantid spermatocytes, the presence of a single mono-oriented X chromosome results in a permanent block to anaphase entry, leading to eventual cell death. However, if such a chromosome is placed under gentle tension by pulling it away from the pole with a microneedle, the cells overcome their metaphase block and proceed through anaphase (*62*). Presumably the external application of tension switches off the "wait anaphase" signal-generating mechanism at the kinetochore.

In cultured vertebrate cells, this "wait anaphase" signal can be transmitted by unattached kinetochores. In the rat kangaroo cell line Ptk_1, attachment of the last free kinetochore to the spindle precedes anaphase onset by about 20 min, and the presence of one or more mono-oriented chromosomes blocks the metaphase-anaphase transition (*63*). However, if the unattached kinetochore of such a mono-oriented chromosome is destroyed with a laser, the cell then enters anaphase ~20 min later (*63*). This was interpreted to show that

destruction of the unattached kinetochore abolished transmission of an inhibitory "wait anaphase" signal.

A fortuitous observation provided the first evidence for a link between the attachment status of a kinetochore and its biochemical makeup. In addition to staining other cellular structures, the phosphoepitope-specific monoclonal antibody 3F3/2 preferentially stains kinetochores that are not under tension (64). Injection of this antibody into cells delays the metaphase-anaphase transition without affecting chromosome movements (65). The kinase-phosphatase balance responsible for expression of this phosphoepitope may either be indicative of, or actually contribute to, the transmission of the "wait anaphase" signal (64, 65).

Recently, a direct experimental link was shown between the expression of the 3F3/2 epitope and kinetochore tension in grasshopper spermatocytes. When bivalents were detached from the metaphase spindle, their kinetochores acquired strong 3F3/2 reactivity. If these bivalents were then directed to reattach to a single spindle pole (no kinetochore tension), 3F3/2 reactivity remained high. However, if such mono-oriented bivalents were then stretched with a microneedle so as to bring one kinetochore under tension, the amount of 3F3/2 epitope at the tense kinetochore underwent a marked decrease (66).

Whereas recent studies have made progress toward proving that kinetochores can send signals and that spindle tension status affects kinetochore biochemistry, fundamental questions remain. On the one hand, studies with 3F3/2 and micromanipulation point to tension as a key factor in the "wait anaphase" signal. On the other hand, the laser ablation studies suggest that kinetochore attachment rather than tension is important. When the unattached kinetochore of a mono-oriented chromosome is ablated, this does not put the attached kinetochore under tension, yet it does turn off the "wait anaphase" signal (63). These inconsistencies may be due to intrinsic differences between meiosis and mitosis (67), or more subtle factors may be involved.

Clearly, no single structure or mechanism can yet be used to define all centromeres. This variability reflects not only the complexity of the functions attributed to the centromere, but also, undoubtedly, the regulatory circuits involved in cell cycle checkpoints that are influenced by the centromere. Continued progress in studying both point and regional centromeres at all stages of the cell cycle should produce a unified view of this important chromosomal structure.

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Chromosomal Control of Meiotic Cell Division

Kim S. McKim and R. Scott Hawley*

Chromosomes have multiple roles both in controlling the cell assembly and structure of the spindle and in determining chromosomal position on the spindle in many meiotic cells and in some types of mitotic cells. Moreover, functionally significant chromosome-microtubule interactions are not limited to the kinetochore but are also mediated by proteins localized along the arms of chromosomes. Finally, chromosomes also play a crucial role in control of the cell cycle.

Chromosomes have long been viewed as simply the cargo of the meiotic and mitotic processes. They were thought to be attached to spindles organized by centrosomes and then dragged back and forth by external forces. Over the last decade, this perception has changed markedly with the realization that chromosomes control much of the cell division process in meiotic cells and sometimes even in mitotic cells. This change of view reflects four recent sets of findings. (i) Molecular motors attached to the kinetochores, and in some cases the chromosome arms, move the chromosomes during mitosis and meiosis. (ii) In many meiotic cells, the divisions take place on acentriolar spindles organized by the chromosomes. (iii) The chromosomes function in error recognition in some meiotic and mitotic cell cycles; that is, cell cycle checkpoints exist that are triggered by abnormalities in chromosome structure or position. (iv) At least in *Drosophila* oocytes, the chromosomal consequences of meiotic crossing-over are crucial for the initiation of a normal arrest in the meiotic cell cycle. These examples may be just the first of many processes in which chromosomes and their substructures control the cell cycle.

Chromosomal Control of Meiotic Spindle Formation

As pointed out by Rieder *et al.* (*1*, p. 187), "the route by which the spindle forms can differ considerably between mitosis and meiosis." Although in most mitotic cells spindle formation is mediated by centrosomes, in some oocytes the spindle appears to be acentriolar and is organized by the chromosomes themselves. A well-studied example of this phenomenon occurs in the oocytes of the fruit fly *Drosophila melanogaster*.

Before metaphase, the chromosomes of *Drosophila* oocytes are located in a single mass known as the karyosome. Meiotic spindle formation begins with the establishment of an array of microtubules lacking a defined pole that emanate from the major chromosomes (Fig. 1) (*2*). As prometaphase continues, these bundles of microtubules are sculpted together on each side of the metaphase plate to form a bipolar spindle. It is often possible to observe that the early spindle is comprised of four sets of microtubule bundles, presumably corresponding to each of the four pairs of homologous chromosomes. The spindle then lengthens and tapers, a mechanism that requires the kinesin-like protein NCD (*3*). The structures that form the poles of these spindles share little in common with mitotic centrosomes, as antibodies that recognize the centrosomal antigens DMAP60, DMAP190, and γ-tubulin do not bind to epitopes within these structures (*3*).

The ability to organize bundles of microtubules into polar spindles is intrinsic to all of the chromosomes in the *Drosophila* oocyte. Individual chromosomes or meiotic bivalents (and, perhaps more specifically, their kinetochores) that have been expelled from the karyosome by a variety of genetic means can assemble small minispindles. In the case of mutations that cause the loss or nondisjunction only of univalent chromosomes (univalents), such as mutations in the *nod* gene, these supernumerary spindles are usually monopolar (*2*). In contrast, in meiotic mutants such as nod^{DTW}, which cause high levels of nondisjunction of chiasmate bivalents (*4*), bipolar arrays are often observed. Although individual chromosomes can organize their own spindles, the formation of either a monopolar or a bipolar spindle depends on whether they possess one (univalent) or two (bivalent) kinetochores.

The ability of chromosomes to organize a functional spindle is not limited to *Drosophila* oocytes. Chromosomes have been shown to organize the spindle in the absence of centrosomes in several meiotic systems (*1*, *5*, *6*). There are also more canonical meiotic systems in which the ability of the chromosomes to organize their own spindle appears to be suppressed by functional centrosomes or in which the chromosomes interact with the centrosomes to form the spindle. For example, in *Drosophila* spermatocytes, a chromosome detached from the canonically centriolar meiotic spindle quickly forms a miniature but functional spindle around itself (*7*). The poles of

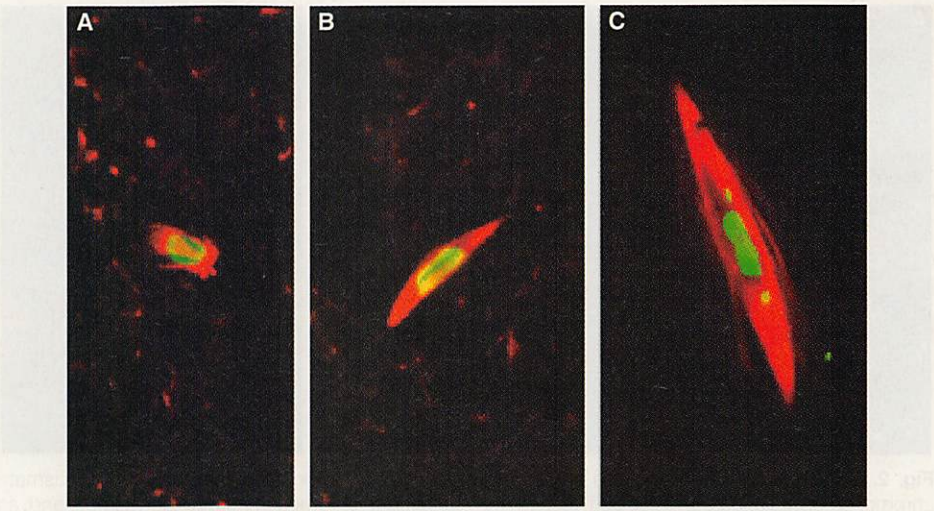

Fig. 1. The normal pathway of spindle assembly in wild-type (+/+) *Drosophila* females. Chromosomes (green) are stained with histone antibody conjugated to fluorescein. Spindles (red) are stained with tubulin antibody conjugated to rhodamine. (**A**) Prometaphase begins with short microtubules that lack a defined pole forming around the karyosome. Pre-assembled spindle microtubules are presumably captured by the chromosomes, stabilizing their plus ends and allowing microtubule elongation. (**B**) The microtubules are then sculpted into a short spindle with defined poles. (**C**) Finally, the metaphase spindle is long and tapered with achiasmate chromosomes precociously moving toward the poles. Meiosis remains arrested at this stage until passage through the oviduct, at which time activation occurs and meiosis is completed.

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these spindles are unrelated to those of the spindle from which the chromosome was detached. To quote Nicklas, "the presence of a chromosome in the cytoplasm not only enhances microtubule assembly but also triggers the formation of a functionally normal spindle, complete with two poles, at a site where a spindle never occurs normally" (*8*, p. 61). Thus, in this case the chromosomes appear to possess the capability to form a spindle on their own, an ability that is actively suppressed in the vicinity of functional centrosomes. Finally, although the chromosomes cannot organize their own spindles without centrosomes in grasshopper spermatocytes, the chromosomes exhibit a size-dependent effect on the structure of the spindle (*9*). That is, the addition of one chromosome near one pole of a newly formed spindle increases the microtubule density in the entire half-spindle fourfold relative to the other half-spindle.

The relation of chromosomes to spindle assembly in mitotic cells is equally complex (*9*). In some cases, such as in echinoderm embryos, the chromosomes cannot organize a spindle in the absence of centrosomes (*10*). There are also systems in which, although the chromosomes cannot organize spindles, they are nonetheless necessary for spindle formation. That is, in echinoderm embryos a pair of centrosomes with microtubule asters cannot produce a spindle in the absence of chromosomes (*9, 11, 12*). Spindle formation in these systems may require specific interactions between the kinetochore and the centrosome. In Chinese hamster ovary cells, kinetochores detached from their chromosomal arms interact with microtubules and together with the centrosomes form a functional spindle (*13*). There are also cases in which chromosomes can organize a spindle when the existing centrioles are unable to do so (*14*), again suggesting that the ability of chromosomes to form spindles is actively suppressed or outcompeted by the presence of functional centrosomes.

Microtubule attachments not limited to kinetochores. The ability of kinetochores to capture microtubule fibers is well documented. This ability presumably extends to diffuse centromeres, to neocentromeres (knobs) in corn, and to facultative centromeres in yeast and humans (*15*). However, at least in meiotic cells, the ability to form stable and functional interactions with microtubules may be a property of chromatin per se and not just of kinetochores or kinetochore-like structures. In *Xenopus* oocytes, chromatin from many sources, including bacteriophage DNA, promotes microtubule assembly. When sperm nuclei with their membranes removed are incubated in extracts of *Xenopus* oocytes arrested at metaphase II, microtubule fibers attach to chromosomes most frequently in regions that lack kinetochores (*12*), which suggests that the ability of chromatin to promote microtubule assembly in meiotic cells is not specific to kinetochores.

The functional significance of such nonkinetochore microtubule attachments along the lengths of chromosome arms is attested to by two sets of findings. First, as indicated above, the movement of chromosomes within a grasshopper spermatocyte spindle alters the distribution of microtubules in a fashion that is proportional to the mass of chromatin and not to the number of kinetochores present (*9*). A spindle was manipulated to have a large bivalent with two kinetochores at one pole and three smaller bivalents with six kinetochores at the other pole. The half-spindle with fewer kinetochores but more chromatin had 30% greater microtubule density than the half-spindle with more kinetochore but less chromatin. Thus, the presence of chromatin had a dramatic effect on spindle structure.

Second, a new class of proteins, the so-called "chromokinesins," have been discovered that localize along the length of chromosome arms and that have important functions in chromosome position, spindle assembly, or both (*16*). The NOD kinesin-like protein, which is found along the length of chromosome arms in *Drosophila* oocytes, positions and holds chromosomes on the developing prometaphase spindle (*17*). In the absence of NOD protein, achiasmate chromosomes are displaced from the developing spindle, apparently by precociously migrating off one of the two ends of the spindle (*2*).

Thus, the NOD protein appears to provide a plateward force that counterbalances the poleward forces acting on the kinetochore; in doing so, NOD substitutes for chiasmata by holding pairs of achiasmate chromosomes at the metaphase plate during spindle assembly. It remains to be determined if NOD performs this function by acting as a plus end–directed motor that actively pushes chromosomes to the plate or, more simply, as a brake that opposes the poleward forces exerted by the kinetochore. A second such chromokinesin, Xklp1, has been identified in *Xenopus* oocytes, which suggests that nonkinetochore microtubule-chromosome interactions may be a feature of oogenesis in a variety of organisms (*18*). This protein appears to be required for maintaining spindle assembly in vitro and in vivo. In oocyte extracts immunologically depleted of Xklp1, bipolar spindles were formed at reduced frequency, and the microtubule density was reduced compared to that in controls. Furthermore, the chromosomes became delocalized and some were released from the spindle, which suggests that Xklp1 may be required for chromosomes to congress at the metaphase plate. Thus, spindle stability may require interactions between nonkinetochore microtubules and Xklp1 on chromosome arms (*12*).

Building a spindle in Drosophila *oocytes may require chromosome opposition*. In *Drosophila* oocytes, spindle assembly requires the proper pairing and alignment of chromosomes, as mediated by either chiasmata or by the NOD kinesin-like protein. That

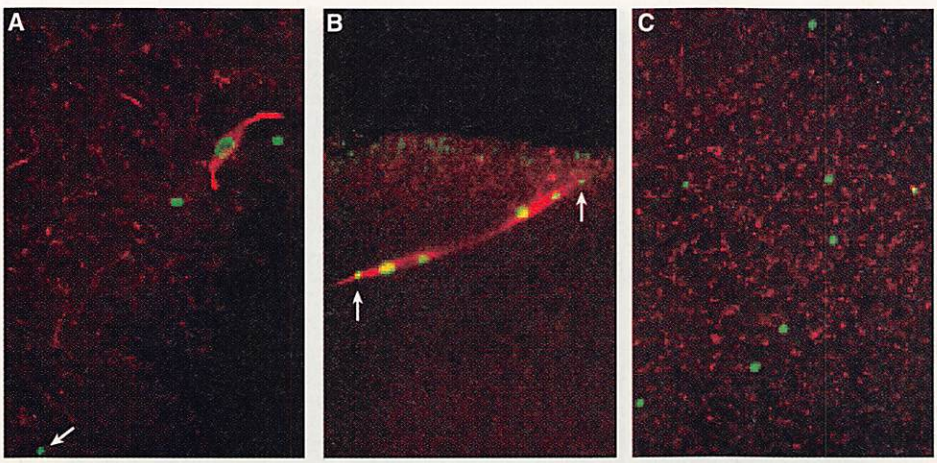

Fig. 2. Spindle formation in *nod* and *mei-218* mutants (*52*). (**A**) In *nod* mutants, only the achiasmate chromosomes are affected. In this oocyte, both the X and fourth chromosomes were achiasmate and, as a result, were ejected from the karyosome. The X chromosome univalents are near the spindle. Shown with an arrow is the single visible fourth chromosome univalent. (**B**) Spindle formation occurs normally in the crossover-defective mutant *mei-218*. In *mei-218* females, the reduction in crossing-over (to less than 10% of that in the wild-type) causes a bypass of metaphase arrest, but this has no effect on spindle assembly or viability (*24*). The two arrows show the small fourth chromosomes leading the larger chromosomes to the poles. (**C**) Spindle formation is severely compromised in the double mutant *nod mei-218*. In these oocytes, both chiasmata and NOD protein are absent. The chromosomes are spread throughout the oocyte, and karyosomes with a mature bipolar spindle are never observed.

is, *Drosophila* oocytes bearing *nod* mutations can assemble a normal spindle in the presence of at least one pair of chiasmate chromosomes (Fig. 2A), and oocytes homozygous for recombination-deficient mutations such as *mei-218* can assemble a normal spindle in the presence of NOD protein (Fig. 2B). However, in double mutant oocytes lacking both chiasmata and NOD protein (*nod mei-218*), mature bipolar spindles are not observed and, instead, the chromosomes are found dispersed throughout the oocyte (Fig. 2C). Bipolar spindle assembly appears defective in these oocytes from the earliest stages of prometaphase; in the double mutant, only the initial stages of bipolar spindle formation are observed.

The requirement for either NOD or at least one chiasma may reflect a need to maintain at least one pair of chromosomes with their kinetochores oriented in opposite directions to provide a central axis for spindle formation. Before nuclear envelope breakdown, homologous chromosomes remain tightly paired, at least in their heterochromatic regions (*19*). Like others, we propose that the kinetochores of each pair of homologous centromeres are usually oriented in opposite directions and thus capture microtubules in such a way as to form a bipolar bundle (*20*). We imagine this kinetochore positioning can be stabilized either by chiasmata or by the NOD kinesin-like protein and that the microtubules captured by the paired kinetochores are sculpted into a bipolar array by the actions of proteins such as the NCD kinesin-like protein. With the spindle formed and the two kinetochores oriented toward opposite poles, the progression of the two chromosomes toward the poles is halted at the metaphase plate by the chiasma, or by the NOD protein in the case of achiasmate chromosomes. This represents a stable position in which the bivalent will remain during metaphase arrest and that provides a framework for spindle formation.

Chromosomal Control of Prometaphase Movement

In centriolar meiotic or mitotic systems, chromosome attachment to the spindle is an error-prone process in which tension on the kinetochore functions as an error-correction mechanism. Because attachment is a stochastic process, at first the chromosomes are usually mono-oriented, such that one or both of the kinetochores are attached to the same pole (Fig. 3). The correction mechanism relies on the fact that these initial kinetochore-spindle attachments are unstable and prone to detachment at the pole. If both kinetochores capture microtubules from the same pole, destabilization and release of an attachment allow for bipolar reattachment. Once the second functional kinetochore captures microtubules from the opposite pole, the microtubule-kinetochore attachment is stabilized and the centromeres congress to the equator (*21*, *22*). Thus, kinetochore-microtubule contacts are tension-sensitive and are stabilized only with bipolar attachment. Moreover, tension on the kinetochore may also control the switching of chromosome movement between poleward and anti-poleward states (*23*). An interesting consequence of having chromosomes organize spindles in meiotic cells is that attaining bipolar attachment is not a problem. The mechanism of spindle formation ensures that the chromosomes are at the metaphase plate with bipolar spindle attachments.

Motors determinining the position of chromosomes. In acentriolar meiotic systems chromosomes begin at the metaphase plate, and thus congression is unnecessary. Nonetheless, multiple mechanisms exist to control the position of chromosomes on these spindles. As the spindle of the *Drosophila* oocyte becomes more elongated, the achiasmate chromosomes move precociously toward the poles, whereas the exchange chromosomes remain locked by their chiasmata at the metaphase plate. The movement of achiasmate chromosomes toward the pole is size-dependent; smaller chromosomes leave the metaphase plate earlier and move farther toward the pole than do larger chromosomes. The timing of movement is clearly a property of size and not a difference between centromeres (*2*). Similarly, in anaphase the smaller chromosomes precede the larger ones to the poles (*24*). This process of size-dependent precocious movement can be explained by the observation that NOD is bound along the entire length of the meiotic chromosomes.

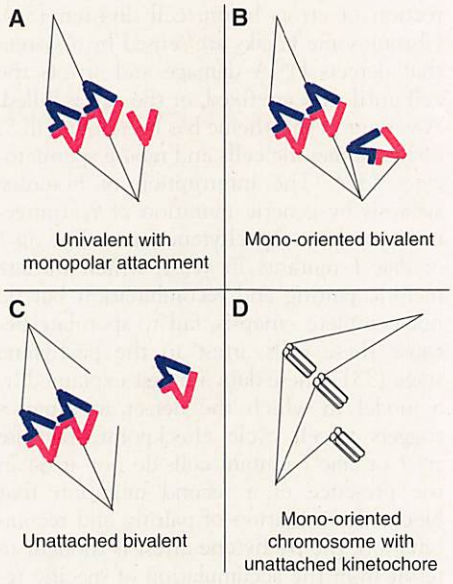

Fig. 3. Four different situations in which meiotic or mitotic chromosomes are misaligned on the spindle. In all four cases, the kinetochores of the misattached chromosomes are not under tension because of the absence of opposing poleward forces. (**A**) A univalent has only one kinetochore and can attach to only one pole. (**B**) Early in spindle attachment a bivalent may have both kinetochores attached to the same pole (mono-oriented). (**C**) A chromosome can become completely detached from the spindle either through micromanipulation or through a failure to attach. (**D**) A mono-oriented chromosome has one kinetochore unattached to spindle fibers. This can occur because the initial attachment of the chromosome to the spindle is often at only one kinetochore. Bipolar orientation is achieved when the other kinetochore makes a spindle attachment.

We propose that each chromosome produces a plateward force in proportion to the number of NOD molecules bound along its length, by acting either as a plus end–directed motor that actively pushes chromosomes to the plate or as a brake that opposes the poleward forces exerted by the kinetochore. Because larger chromosomes bind more NOD protein, they might be expected to exert a larger plateward force than smaller chromosomes, thus explaining the fact that larger chromosomes remain closer to the metaphase plate than smaller chromosomes. The process of size-dependent positioning of chromosomes on spindles is not limited to meiotic cells. Severing the arms off of newt mitotic chromosomes results in the kinetochore-bearing fragment moving closer to the pole and the severed arm moving to a position farther from the pole (*22*). Thus, the "chromokinesins" may be a component of the polar ejection force.

Kinetochore-Mediated Control of Anaphase Chromosome Movement

For many years, the prevailing model for anaphase chromosome movement was known as the "traction hypothesis" in which chromosomes were reeled in to the poles at anaphase by forces acting at the poles or along the kinetochore microtubules. More recently, it has been found that molecules within the kinetochores provide forces for chromosome movement to the poles. These experiments have been reviewed extensively elsewhere (*8*, *25*). Here, we focus on the degree to which structures within the kinetochore control its meiotic behavior.

Determination of reductional or equational segregation. During meiosis in *Drosophila*, chromosomes conduct two different types of division. In meiosis I (reductional), the sister kinetochores stay together while the homologs segregate. In meiosis II (equational), the sister kinetochores segregate like in a mitotic division. The determinants of

whether a given chromosome will segregate reductionally or equationally reside within the kinetochores rather than on the spindle. This conclusion is derived from an elegant experiment in which two spermatocytes from the grasshopper *Dissosteira carolina* were fused, one at the first meiotic metaphase, the other in the second meiotic metaphase (*26*) (Fig. 4). A bivalent was detached from the meiosis I spindle and allowed to reattach to the adjacent meiosis II spindle. At anaphase II, the displaced bivalent still segregated reductionally (that is, the two homologs comprising this bivalent moved to opposite poles), despite its location on a spindle where all the other chromosomes segregated equationally. Thus, the instructions for proper behavior at meiosis I reside within the chromosome, not in the spindle.

That these instructions lie within the kinetochores and are not general properties of the chromosomes is suggested by work on mutations that produce an apomictic meiosis (meiosis with a single division) in the yeast *Saccharomyces cerevisiae* (*27*). During this division, the segregation behavior of individual chromosomes is autonomous and is determined by the centromeres they carry; some centromeres show a preference for reductional instead of equational segregation, or vice versa. The propensity for reductional or equational segregation is maintained by a given centromere even when it is moved to a different chromosome (replacing the existing centromere). The differential behavior of centromeres within a single meiotic division also occurs in several insect species, when during the first meiotic division the sex chromosomes segregate equationally, whereas the remainder of the complement segregate reductionally (*28*).

Differences in centromere sequences might also be expected to result in different force-generating characteristics at the kinetochore. For example, the amount of force pulling a chromosome to the poles could be modulated by microtubule-kinetochore interactions. This is suggested by cytological analysis of trivalents in praying mantid and grasshopper spermatocytes (*29*), where one kinetochore is oriented toward one pole and the other two kinetochores are oriented to the opposite pole. When trivalents are newly made with radiation, their positions on the spindle are consistent with the effects of having two kinetochores exert a stronger poleward force than a single kinetochore applying force in the opposite direction. That is, their stable position at the metaphase plate is closer to the pole to which the two kinetochores are attached.

In contrast, "natural" trivalents take a more central position at the metaphase plate, despite the fact that the force of two kinetochores is opposed to a single kinetochore pulling in the opposite direction. This suggests that together the two kinetochores moving in the same direction pull with a force that is equal to the one kinetochore pulling in the opposite direction. This behavior might reflect the ability of motors at one kinetochore to pull with only enough force to oppose the forces pulling in the opposite direction. Alternatively, different centromeres, by virtue of their DNA sequences, might build kinetochores of different strength. The possibility that different centromeric regions might differ in their ability to exert force was also suggested in studies on the meiotic behavior of dicentric chromosomes in *Drosophila* oocytes (*30*). The segregational behavior of these dicentrics differed substantially depending on the heterochromatic content of the two chromosomes involved in the dicentric chromosome.

Chromosome-Mediated Cell Cycle Control

In most cases, chromosome damage or aberrant chromosome behavior signals an arrest in the cell cycle in order to allow the correction of errors before cell division (*31*). Chromosome breaks are sensed by a system that detects DNA damage and arrests the cell until they are fixed, or the cell is killed. A variant in this theme has been found in *S. cerevisiae* meiotic cells and mouse spermatocytes (*32*). The interruption of homolog synapsis by genetic mutation or rearrangement results in a pachytene arrest. The *zip-1* or *dmc-1* mutants in yeast, which initiate meiotic pairing and recombination but do not complete synapsis, fail to sporulate because these cells arrest in the pachytene stage (*33*). These data are best explained by a model in which the defect in synapsis triggers a cell cycle checkpoint. Because *zip-1* or *dmc-1* mutant cells do not arrest in the presence of a second mutation that blocks the initiation of pairing and recombination, the pachytene arrest is thought to result from the accumulation of specific recombination intermediates that trigger the checkpoint. Alternatively, the checkpoint might be triggered by a meiosis-specific mechanism that senses the failure or deterioration of synapsis.

Triggering of meiotic or mitotic arrest by single, unattached chromosomes. Unrepaired DNA breaks are not the only chromosomal anomalies that can signal a problem in the cell cycle. Checkpoints monitor the completion of chromosome alignment during metaphase in meiotic and mitotic cells (*34*) (Fig. 3). In mitotic Ptk cells (*35*), the time between nuclear envelope breakdown and anaphase is longer in cells with as few as one kinetochore unattached to microtubules. The delay in metaphase allows time for all kinetochores to attach to microtubules. The failure to prevent division before such errors are corrected would result in nondisjunction and aneuploidy. Similarly, in praying mantid spermatocytes a single univalent with a mono-oriented kinetochore can signal a fatal prolongation of metaphase (*36*). In this situation, the delay leads to the degeneration of cells that otherwise would produce chromosomally abnormal sperm. The studies summarized below reveal that the location of the signal that triggers this checkpoint is at the kinetochore, that the stimulus of the signal is tension, and that the chemical signal involves the phosphorylation of kinetochore proteins.

The arrest signal originates at the kinetochore. Three lines of evidence suggest that bipolar kinetochore attachment to the spindle may be crucial for the metaphase-anaphase transition in some meiotic and mitotic cells. (i) Mutation of the yeast centromere leads to a prolongation of mitosis (*37*). (ii) The injection of centromere protein antibodies into G_2 cells does not disrupt spindle assembly but causes metaphase arrest (*38*); this suggests either that centromeric proteins are involved in signaling the metaphase-to-anaphase transition or that proper kinetochore function is monitored by a checkpoint. (iii) Most directly, when

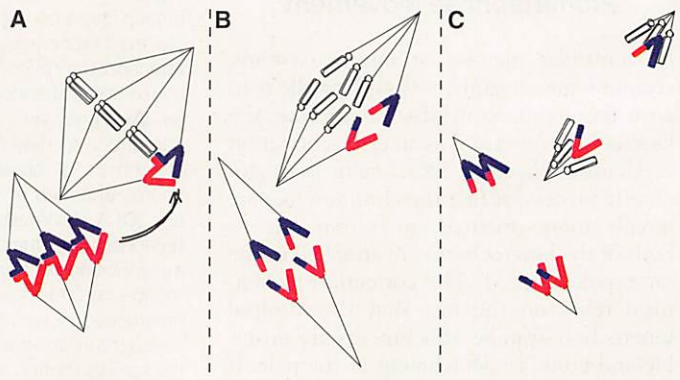

Fig. 4. The results of moving a meiosis I chromosome onto a meiosis II spindle. A schematic diagram showing two spindles; on the left is a meiosis I spindle, and on the right is a meiosis II spindle. (**A**) A bivalent is moved by micromanipulation from the meiosis I spindle onto the adjacent meiosis II spindle. (**B** and **C**) This chromosome orients and segregates reductionally, even though all the other chromosomes on the spindle segregate equationally.

the unattached kinetochore of a mono-oriented chromosome in mitotic Ptk cells was destroyed by laser ablation, it was no longer able to delay the metaphase-to-anaphase transition (*39*). Thus, the unattached kinetochore of a mono-oriented chromosome inhibits the transition into anaphase.

Transition delay by absence of tension on the kinetochore. Tension arises when a properly oriented chromosome possesses microtubule attachments to opposing poles. During meiosis, for example, each kinetochore of the bivalent applies a poleward force as it moves along kinetochore microtubules. In a normal bivalent, these poleward forces are opposed and balanced by the chiasmata, resulting in tension on both kinetochores. The failure to maintain tension can lead to cell death in some cell types. In praying mantid spermatocytes a single mono-oriented univalent signals a prolongation of metaphase, leading to apoptosis (*36*). In these cells, three sex chromosomes must remain connected as a trivalent to orient at meiosis I. In some cases, one sex chromosome becomes separated from its two partners and finds itself connected to only one pole. In these cells, the metaphase-to-anaphase transition is delayed and eventually leads to cell death (*36*). The role of tension in this system was tested by pulling on the mono-oriented sex chromosome with a micromanipulation needle to simulate the bipolar tension experienced by this chromosome when it is part of a trivalent at the metaphase plate (*36*). When tension was applied, anaphase initiated on schedule. Thus, the lack of tension on a kinetochore signaled the delay in the metaphase-to-anaphase transition.

Absence of tension and change in protein phosphorylation. Tension on the kinetochores can generate a checkpoint signal, but the chemical nature of this signal is not known. A candidate kinetochore signaling protein was detected with an antibody, 3F3, that reacts strongly with some kinetochore proteins when they are phosphorylated. 3F3 reacts strongly with kinetochores before they are attached to the spindle, but the reaction weakens as the chromosomes attach to the spindle and move to the metaphase equator (*40*). Early in grasshopper spermatocyte spindle formation, the kinetochores of mono-oriented bivalents are intensely stained by the 3F3 antibody. Because both kinetochores of the bivalents are attached to the same pole, the kinetochores are not under tension. Thus, some kinetochore proteins are phosphorylated when not under tension (*41*). Individual chromosomes were detached from the meiotic spindle with micromanipulation techniques (*41*). Both kinetochores of these bivalents lacked tension for as long as they were held off the spindle by micromanipulation. When a chromosome was detached for only 5 min, no increase in 3F3 staining was observed. Longer detachment, however, resulted in twofold greater staining of the kinetochore with 3F3. The time required for phosphorylation of the kinetochore in response to "relaxation" of kinetochore forces was between 5 and 10 min (*41*).

These principles can be illustrated by analysis of bivalents positioned near one pole that had been manipulated to have both kinetochores attached to it (*41*) (Fig. 3). Without force exerted toward the opposite pole, there was no tension on either kinetochore of the bivalent, and both fluoresced brightly with 3F3. When tension was applied to only one of the two kinetochores of this bivalent (by inserting a microneedle into the bivalent and pulling one of the two kinetochores toward the equator), the kinetochore placed under tension quickly exhibited reduced staining to a level similar to that seen in the other properly oriented chromosomes in the same cell. The other kinetochore of the same bivalent, which lacked tension, retained its bright 3F3 staining, clearly showing that the tension signal is localized to a specific kinetochore.

Although there is no direct link between the phosphorylation state of the kinetochore and the cell cycle arrest, the correlations are strong (*41*). First, misattached chromosomes have phosphorylated kinetochores and trigger a metaphase checkpoint. Second, both the triggers of the checkpoint and the changes in kinetochore phosphorylation respond to tension and operate at the kinetochore. It is unknown what proteins are recognized by the 3F3 antibody and if they directly mediate the tension signal. Finding these proteins will provide insight into how a physical stimulus such as tension can change kinetochore chemistry and how the signal is transduced from the kinetochore proteins to the cell cycle controls. The immunolocalization of 3F3 (*42*) shows that the tension-sensitive proteins are found in the middle layer of the kinetochore, among the struts between the inner and outer plates. This is a logical location for proteins that detect tension or stretching of the kinetochore.

Not all kinetochores that lack tension produce a checkpoint signal. In several organisms, univalent X chromosomes can congress to the metaphase plate in the absence of a homolog (*43*). In *Caenorhabditis elegans* X/O males, univalent X chromosomes also congress to the metaphase I plate in the absence of a homolog (*6*). This is a specific property of the X chromosome and not of univalents in general. During meiosis, autosomal univalents behave abnormal-

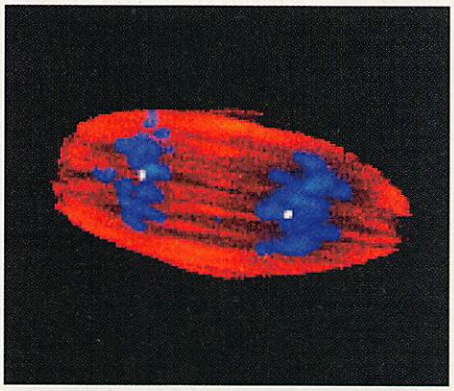

Fig. 5. Anaphase I mouse oocyte showing equational segregation of the univalent X chromosome. The spindle, in red, was detected by rhodamine-labeled antibody. The chromosomes (blue) are stained with TO-PRO, and the X chromosome (white) is detected by a fluoroscein isothiocyanate–labeled probe.

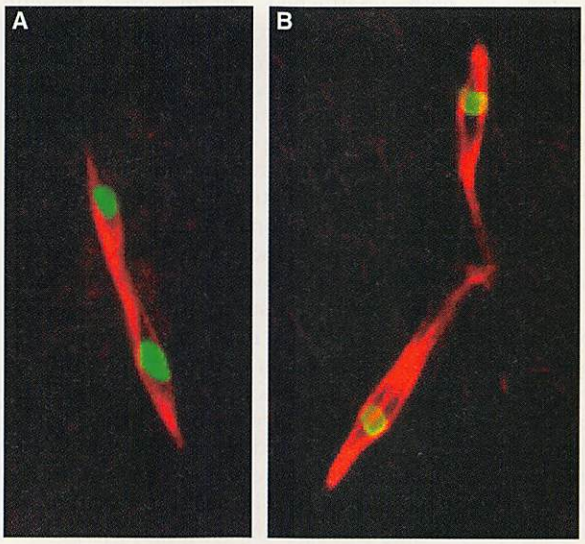

Fig. 6. Mature (stage 14) oocytes in the recombination-defective mutant *mei-W68* (*52, 53*). Meiosis normally arrests at metaphase I in stage 14 oocytes (see Fig. 1). However, in *mei-W68* mutants, crossing-over and thus chiasmata are eliminated. The result is a loss of kinetochore tension and a bypass of metaphase arrest. Shown here are two stage 14 oocytes with post-metaphase I stages: (**A**) anaphase I and (**B**) metaphase II. Similar results were seen with other meiotic mutants (such as *mei-218*) (Fig. 2).

ly, resulting in chromosome misdivision, loss, or equational segregation (*44*) (Fig. 5). Similarly, in the grasshopper spermatocytes described above, the univalent X chromosome does not delay anaphase—otherwise, no sperm could be made. Nicklas *et al.* (*41*) suggested that these cells must have evolved a mechanism to suppress checkpoint signals from the naturally univalent X chromosome. Consistent with this hypothesis, the kinetochore of the grasshopper X chromosome never stains strongly with 3F3. Thus, the kinetochores of these chromosomes are prevented from signaling metaphase arrest, allowing them to congress and segregate normally.

Kinetochore tension in Drosophila *oocytes.* Tension can be used for different purposes in different cell types. Tension on the kinetochore is a signal in *Drosophila* females to arrest meiosis at metaphase I. Anaphase I does not normally begin until the oocyte passes through the oviduct, at which time sister chromatid cohesion distal to the chiasmata is released (Fig. 1). In oocytes with a drastic reduction in crossing-over as a result of recombination-defective mutations, metaphase I arrest was abolished and anaphase and meiosis II figures were observed (Fig. 6) (*24*). Thus, crossing-over and the resulting chiasmata are required for metaphase arrest. Without chiasmata, all chromosomes would have microtubule attachments to only one kinetochore, which suggests that the balancing of kinetochore forces by chiasmata was the signal for metaphase arrest.

This hypothesis was tested with female fruit flies whose normal karyotype consisted entirely of "compound" chromosomes, in which each pair of homologous chromosome arms is attached to a single centromere (*45*). These chromosomes allow crossing-over to occur, but they do not induce tension on the kinetochores because the chiasmata do not connect centromeres on different chromosomes. As in the recombination-defective mutants, metaphase I arrest was abolished in these females. That the anaphase I and meiosis II figures observed in these two experiments represent the normal continuation of the meiotic process is shown by the fact that eggs produced by these females are fully capable of producing viable offspring upon fertilization by sperm of the correct genotype.

In a separate experiment, females were created in which all but one pair of chromosome arms were arranged as compound chromosomes (*45*). In these females, the one normal pair of chromosomes was able to form chiasmata that joined two centromeres and metaphase arrest was virtually always observed. Similarly, some recombination-defective mutants have a low level of crossing-over, such that approximately 25% of the mutant oocytes possess at least one chiasma. In these mutants, a similar frequency of oocytes that were arrested at metaphase I was observed, which suggests that tension from even a single chiasmate bivalent could induce metaphase arrest (*24*).

There is a crucial difference between the situation in *Drosophila* oocytes and the other two systems mentioned here (mitotic Ptk cells and insect spermatocytes). In *Drosophila* females, tension is a signal that causes metaphase arrest. In the other two systems, it is the lack of tension that causes metaphase arrest. Thus, the transduction of the tension signal has been modified in these systems for specific needs. In the spermatocytes and Ptk cells, the lack of tension is a signal that there is a problem in aligning the chromosomes on the spindle and that anaphase needs to be delayed until the problem is fixed (*39*) or until the cell degenerates (*36*). In *Drosophila* females, metaphase I arrest is the normal course of events, and thus chromosome misalignment may not be a problem. In both systems, the signal from a single chromosome or bivalent can induce cell cycle arrest. It is probably easier for the cell to respond to one misaligned chromosome (or to one chiasmate bivalent in *Drosophila* oocytes) than it is to detect small changes in the number of aligned chromosomes (*39*, *46*).

The absence of a chromosome misalignment checkpoint during *Drosophila* female meiosis (*47*) is striking. Meiosis proceeds without an arrest with any number of univalent chromosomes or disorganization of the spindle (Fig. 2), which demonstrates that there is no checkpoint for mono-oriented or misaligned chromosomes in *Drosophila* female meiosis. Some embryonic systems also lack checkpoints, which is perhaps a compromise to allow rapid cell division (*31*). For example, sea urchin zygotes also proceed into anaphase with unattached chromosomes present (*48*). A similar argument can apparently be made for oocytes in X/O mouse females. In this case, oocytes enter anaphase with a single unpaired X chromosome and without signaling a metaphase I checkpoint. Furthermore, the univalent segregates either equationally or reductionally at the first division, which shows that the nature of the spindle attachment (bipolar or monopolar) does not effect the metaphase-to-anaphase transition (*47*) (Fig. 5). Conceivably, most or all oocyte systems lack a system to detect mono-oriented chromosomes on the meiotic spindle. This may be related to the fact that, unlike spermatocytes and yeast cells, most oocytes naturally arrest at some point during meiosis I or II. The lack of a metaphase checkpoint in human oocytes may cause the very high frequency of meiotic errors in human oocytes as compared to those in sperm (*47*).

Summary

The studies described above point to the existence of multiple roles for chromosomes in the processes of spindle assembly and maintenance and in positioning chromosomes on the meiotic metaphase spindle. We have also pointed out the role of chromosomes, and more specifically that of kinetochores, both in triggering pre-programmed cell cycle arrests and in signaling unplanned arrest in response to mitotic or meiotic errors. Taken together, these studies indicate that chromosomes are not passive gene carriers of the cell division process, but complex organelles that possess both motor activities and the capacity to initiate and respond to cell cycle controls.

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15. In holo-kinetic organisms (*28*), any DNA sequence appears to promote kinetochore assembly, and thus spindle attachment occurs along the entire arm of the chromosomes in mitotic cells. That is, the kinetochores of these organisms are built along the entire length of the chromosomes. In such organisms [*C. elegans* is one of the best studied examples (*49*)], any piece of DNA introduced into the genome possesses the ability to form microtubule attachments that ensure proper segregation. Moreover, some chromosomes contain regions that act like conditional centromeres; under some conditions, they will bind microtubules and function like kinetochores. A classical example is the knob10 (K10) element in maize (*50*). In the presence of a crossover between K10 and the centromere, K10 will bind microtubules and lead the half-bivalent to the pole. Because the leading chromatids at anaphase end up in the gamete, the conditional use of K10 as a microtubule-binding region increases

the chance that K10 will be passed on to the next generation. More recent examples have been found with *Schizosaccharomyces pombe* plasmids carrying portions of a canonical centromere and human marker chromosomes (*51*). In all three cases, epigenetic modifications may control the centromeric activity of these regions.
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Telomeres: Beginning to Understand the End

Virginia A. Zakian

Telomeres are the protein-DNA structures at the ends of eukaryotic chromosomes. In yeast, and probably most other eukaryotes, telomeres are essential. They allow the cell to distinguish intact from broken chromosomes, protect chromosomes from degradation, and are substrates for novel replication mechanisms. Telomeres are usually replicated by telomerase, a telomere-specific reverse transcriptase, although telomerase-independent mechanisms of telomere maintenance exist. Telomere replication is both cell cycle– and developmentally regulated, and its control is likely to be complex. Because telomere loss causes the kinds of chromosomal changes associated with cancer and aging, an understanding of telomere biology has medical relevance.

Eukaryotes have linear chromosomes, and the ends of these linear chromosomes are composed of protein-DNA structures called telomeres. Telomeres were first characterized in ciliated protozoans such as *Tetrahymena* (*1*) and *Oxytricha* (*2*, *3*). After meiosis, in a developmentally regulated process, ciliate chromosomes are broken up into subchromosomal sized fragments. These fragments are replicated to generate a polyploid nucleus, the macronucleus, which can contain literally millions of telomeres (reviewed in *4*). Thus, compared to the modest number of chromosomes, and hence telomeres, in most organisms, the ciliate macronucleus is a rich source of both telomeric DNA and the structural proteins and enzymes that protect and replicate this DNA. In spite of the structural novelty of the ciliate macronucleus, many features of telomeres first discovered in ciliates are also true of telomeres in organisms like *Saccharomyces* and humans, both of which have conventional chromosomes. Indeed, telomeres display considerable conservation of both structure and function from single-celled organisms to higher plants and animals, as well as some intriguing interspecies differences.

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Telomeric DNA

In most organisms, telomeric DNA consists of a tandem array of very simple sequence DNA (Table 1) (*1–3*, *5–28*). Most telomeric repeat sequences are short and precise. For example, telomeric DNA in *Tetrahymena* is comprised of the 6-bp (base pair) sequence C_4A_2/T_2G_4. However, some telomeric sequences are heterogeneous (for example, $C_{1\text{-}3}A/TG_{1\text{-}3}$ in *Saccharomyces*) and in some, the repeat unit is considerably longer (for example, 25 bp in *Kluyveromyces lactis*). Moreover, *Drosophila* has a completely different and so far novel telomere structure. Rather than simple repeats, the DNA at the ends of *Drosophila* chromosomes is composed of a transposable element (*29–31*).

In most organisms, the subtelomeric regions immediately internal to the simple repeats consist of middle repetitive sequences, called telomere-associated (TA) DNA, which bear a superficial similarity to the transposons at the ends of *Drosophila* chromosomes. In *Saccharomyces*, there are two classes of TA elements, X and Y′ (*32*), one or both of which are found on most or all telomeres (Fig. 1). The array of TA DNA at a given chromosome end can expand and contract. However, in those or-

ganisms in which these changes have been studied, they occur by recombination, not transposition (*33*, *34*). Because a yeast chromosome without TA DNA replicates, segregates, and recombines in mitosis and meiosis in a manner indistinguishable from that of the same chromosome with TA DNA at both ends, yeast TA DNA is dispensable under normal growth conditions (*35*). Here, telomeric DNA refers only to the repeats at the very ends of chromosomes.

The sequences of telomeric DNA from a wide variety of organisms have been determined (Table 1). Because multiple telomeric sequences are found, telomere function does not require a unique DNA sequence. Moreover, in many organisms, telomeric DNA is also found at internal sites on the chromosome (for example, *36*–*38*), which demonstrates that sequence alone does not make a telomere. Although there is considerable diversity among the different telomeric DNAs, even very distantly related organisms can have the same telomeric sequence. For example, C_3TA_2/T_2AG_3 is the sequence of telomeric DNA in all vertebrates, the protozoan *Trypanosoma*, and several slime molds and fungi (Table 1). In other cases, different organisms have a different telomeric sequence, but the two sequences are clearly related, like the telomeric sequences of the distantly related ciliates *Tetrahymena* and *Oxytricha*, which are, respectively, C_4A_2/T_2G_4 and C_4A_4/T_4G_4. As more telomeric sequences become known, it becomes more difficult to identify even a loose consensus sequence to describe them. For all known telomeric DNA sequences except that of *Parascaris*, there is a strand bias in the various telomeric DNAs such that the strand running 5′ to 3′ from the center toward the end of the DNA molecule has more G residues than its complement and, further, the G's in this strand are clustered.

In the macronuclear DNA of ciliated protozoans, the G-rich strand is extended to form a 12- to 16-base single-strand G tail that exists throughout most or all of the cell cycle (*2*, *3*). G tails can also be detected on *Saccharomyces* chromosomes, but they are longer and transient, appearing late in the S phase at the same time that telomeric DNA is replicated (*39*, *40*). In vitro, the G strand of telomeric DNA can form a variety of non–Watson-Crick base-paired structures, including four-stranded helices or G quartets held together by multiple G–G base pairs (*41*–*43*). Although it is not clear if these stable DNA structures exist in vivo, the single-strand G tails on *Saccharomyces* chromosomes allow two telomeres to interact, apparently by non–Watson-Crick base pairing (*40*). If DNA structures based on G–G base pairs are important for some aspect of telomere function, it would explain the prevalence of tandem G residues in telomeric DNA.

The average amount of telomeric DNA at a chromosome end varies from organism to organism. For example, mice have as much as 150 kb of telomeric DNA per telomere (*44*, *45*), whereas telomeres on *Oxytricha* macronuclear DNA molecules are only 20 bp in length (*3*). Moreover, in contrast to *Oxytricha* and related ciliates, in which all macronuclear telomeres are the same discrete size, in all other organisms the amount of telomeric DNA per telomere fluctuates. For example, the amount of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA at individual yeast telomeres in a wild-type strain ranges from ~200 to ~400 bp, and this amount increases and decreases stochastically (*46*). Heterogeneity and spontaneous changes in telomere length probably reflect a complex balance between processes that lead to degradation and those that lengthen telomeric tracts. In addition to this inherent length heterogeneity, the average telomere length in yeast and other organisms can increase or decrease in response to genetic (*47*) or nutritional (*48*) changes. In some organisms, like *Tetrahymena* (*49*) and *Trypanosoma* (*50*), the average telomere length continuously increases during log-phase growth. In contrast, in human somatic cells, telomeres slowly shrink during successive cell divisions (*51*, *52*). The inherent heterogeneity characteristic of virtually all telomeric DNAs suggested that telomeres are not maintained by a conventional replication process. Further, the fluid nature of telomeric and subtelomeric regions suggests

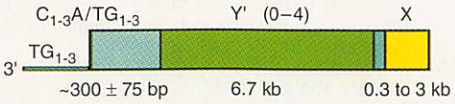

Fig. 1. Structure of a *Saccharomyces* telomere (not drawn to scale). Each yeast chromosome begins and ends with ~300 bp of duplex $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric DNA with the exact length varying from telomere to telomere. Single-strand $TG_{1\text{-}3}$ tails of >30 bases are detected transiently at the end of the S phase (*40*). It is not known if shorter $TG_{1\text{-}3}$ tails exist at other times in the cell cycle. About two-thirds of the telomeres bear one to four copies of the 6.7-kb Y′ element or a 5.2-kb deletion derivative of Y′ (*32*). X, the middle repetitive DNA element internal to Y′, is heterogeneous and comprised of several small repeats, some of which are found on most chromosomes (*32*, *147*). Internal stretches of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA of ~50 to 130 bp are often found between tandem Y′ elements or between X and Y′ (*36*).

Table 1. Sequences of telomeric DNAs. The sequence of the strand running 5′ to 3′ from the end of the molecule forward to its center is presented first.

Organism	Sequence	Reference
Protozoa		
Tetrahymena	C_4A_2/T_2G_4	(*1*)
Paramecium	$C_{3A}^{C}A_{2/2T}^{G}G_3$	(*5*)
Oxytricha	C_4A_4/T_4G_4	(*2*, *3*)
Plasmodium	$C_3T_G^AA_2/T_{2C}^TAG_3$	(*6*)
Trypanosoma	C_3TA_2/T_2AG_3	(*7*, *8*)
Giardia	C_3TA/TAG_3	(*9*)
Slime molds		
Physarum	C_3TA_2/T_2AG_3	(*10*)
Didymium	C_3TA_2/T_2AG_3	(*10*)
Dictyostelium	$C_{1\text{-}8}T/AG_{1\text{-}8}$	(*11*)
Fungi		
Saccharomyces	$C_{2\text{-}3}ACA_{1\text{-}6}/T_{1\text{-}6}GTG_{2\text{-}3}$	(*12*, *13*)
Kluyveromyces	$ACAC_2ACATAC_2TA_2TCA_3TC_2GA/TCG_2AT_3GAT_2AG_2TATGTG_2TGT$	(*14*)
Candida	$ACAC_2A_2GA_2GT_2AGACATC_2GT/ACG_2ATGTCTA_2CT_2CT_2G_2TGT$	(*15*)
Schizosaccharomyces	$C_{1\text{-}6}G_{0\text{-}1}T_{0\text{-}1}GTA_{1\text{-}2}/T_{1\text{-}2}ACA_{0\text{-}1}C_{0\text{-}1}G_{1\text{-}6}$	(*16*)
Neurospora	C_3TA_2/T_2AG_3	(*17*)
Podospora	C_3TA_2/T_2AG_3	(*18*)
Cryptococcus	$A_2C_{3\text{-}5}T/AG_{3\text{-}5}T_2$	(*19*)
Cladosporium	C_3TA_2/T_2AG_3	(*20*)
Invertebrates		
Caenorhabditis	GC_2TA_2/T_2AG_2C	
Ascaris	GC_2TA_2/T_2AG_2C	(*21*)
Parascaris	$TGCA_2/T_2GCA$	(*22*)
Bombyx, other insects	C_2TA_2/T_2AG_2	(*23*)
Vertebrates		
	C_3TA_2/T_2AG_3	(*24*)
Plants		
Chlamydomonas	C_3TA_4/T_4AG_3	(*25*)
Chlorella	C_3TA_3/T_3AG_3	(*26*)
Arabidopsis	C_3TA_3/T_3AG_3	(*27*)
Tomato	$C_3A_A^TT_2/A_{2T}^ATG_3$	(*28*)

that telomere function does not require a precise protein-DNA structure. For example, *Saccharomyces tel1* strains have telomeres about a third of the size of those in wild-type cells (*47*), although they have chromosome loss rates close to those of the wild type (*53*) and presumably relatively normal telomere function.

Telomere Structural Proteins

Much less is known about the structural proteins that interact with telomeric DNA than about the DNA itself. The telomeric DNA at the ends of *Saccharomyces* chromosomes (*54*) and on ciliate macronuclear DNA molecules (*55*, *56*) is found in a non-nucleosomal but discrete chromatin structure, called the telosome, which encompasses the entire terminal array of telomeric repeats. Although the DNA adjacent to the *Saccharomyces* telosome is packaged in nucleosomes (*54*), these nucleosomes differ from those in most other regions of the yeast genome, having features (such as hypoacetylated histones) that are characteristic of transcriptionally inactive chromatin (*57*). In mammals, whose telomeres are much longer than those of yeasts or ciliates, most of the simple repeated telomeric DNA is packaged in closely spaced nucleosomes (*58*, *59*). However, the telomeric repeats at the very ends of human chromosomes are found in a telosome-like structure (*59*). Thus, at least some features of the chromatin structure of telomeric regions are conserved between lower and higher eukaryotes.

The major structural protein in the *Saccharomyces* telosome is the product of the essential gene *RAP1* (*60*, *61*). In vitro the *RAP1* protein, Rap1p, binds with high affinity (*62*) to the many recognition sites distributed in tandem throughout the length of a telomere (*63*). Both in vivo and in vitro Rap1p binding is not limited to telomeres. When this multifunctional protein binds at an internal site on the chromosome, it can act either as a transcriptional activator or as a transcriptional repressor (for example, *64*, *65*). Rap1p also binds to single-strand $TG_{1\text{-}3}$ DNA in vitro but with an affinity several orders of magnitude less than that for duplex telomeric DNA (*66*).

Telosomal proteins have also been identified in *Oxytricha* (*67*). The genes encoding the *Oxytricha* proteins have no sequence similarity to *RAP1* nor to any of the other proteins thought to be minor constituents of the yeast telosome (*68*, *69*). Binding of the *Oxytricha* proteins requires the single-strand $T_4G_4T_4G_4$ tail found at each end of all macronuclear DNA molecules (*67*). Thus, unlike Rap1p, the *Oxytricha* proteins will not bind to internal tracts of telomeric sequence. Because the binding of the *Oxytricha* telosomal proteins is limited to the very end of the chromosome, these proteins are terminus-specific DNA binding proteins. One of the two *Oxytricha* telosomal proteins facilitates the formation of G quartet DNA in vitro (*70*), providing strong circumstantial support for the presence of four-stranded DNA in vivo. Although G strand–specific binding proteins have been identified in other organisms, including yeast (*71*), the *Oxytricha* protein is the only one known to be localized to telomeres in vivo. Indeed, many of the abundant proteins that display G strand–specific binding in vitro are probably RNA binding proteins in vivo (*71–73*). However, genetic data suggest that a terminus-specific binding activity is present in *Saccharomyces* (*74*). In addition, an activity has been detected in *Xenopus* extracts that, like that of the *Oxytricha* proteins, displays terminus-specific binding in vitro (*75*). Thus, terminus-limited binding proteins may be a general feature of telomeric chromatin.

Telomere Functions

Telomeres were first defined functionally in *Drosophila*. Muller argued that the inability to recover *Drosophila* chromosomes without an end after x-ray–induced chromosome breakage indicated that the telomere was essential (*76*). McClintock reported that broken chromosomes in maize frequently fuse with other broken chromosomes to generate dicentric chromosomes, destined for breakage in a subsequent cell division. Because chromosomes with telomeres did not fuse with one another, she hypothesized that the telomere's essential function can be explained by its role in protecting chromosomes from end-to-end fusions (*77*, *78*).

The biochemical properties of DNA polymerases suggested another essential function for telomeres (*79*) (Fig. 2). Conventional DNA polymerases replicate DNA only in the 5′ to 3′ direction and cannot initiate synthesis of a DNA chain de novo. The DNA polymerases that replicate eukaryotic chromosomes use an 8- to 12-base stretch of RNA to prime DNA synthesis. As a consequence, after DNA replication one end of a linear chromosome will be replicated to the very end, whereas the other end will have a short 8- to 12-base gap generated by removal of the RNA primer (Fig. 2). Because this 5′ gap cannot be filled in by a conventional DNA polymerase, in every other cell division a given DNA end will be incompletely replicated. Hence, the end of a linear chromosome will shorten by an average of 4 to 6 bases per cell division unless telomeres act as substrates for an alternative replication mechanism.

By removing the telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract from one end of a dispensable chromosome, it was possible to determine directly the fate of a yeast chromosome after telomere loss (*80*). A chromosome end without a telomere is progressively lost (*80*, *81*). Because this loss occurs in the absence of cell division and at a rate much faster than that expected for incomplete replication, it must be the result of degradation (*80*, *82*). Thus, yeast telomeres are essential for chromosome integrity. The ability of telomeres to protect DNA ends from degradation can be mimicked in vitro. The presence of the *Oxytricha* telosomal proteins on otherwise naked macronuclear DNA molecules prevents their exonucleolytic degradation (*67*). The importance of telomeres in preventing degradation can also

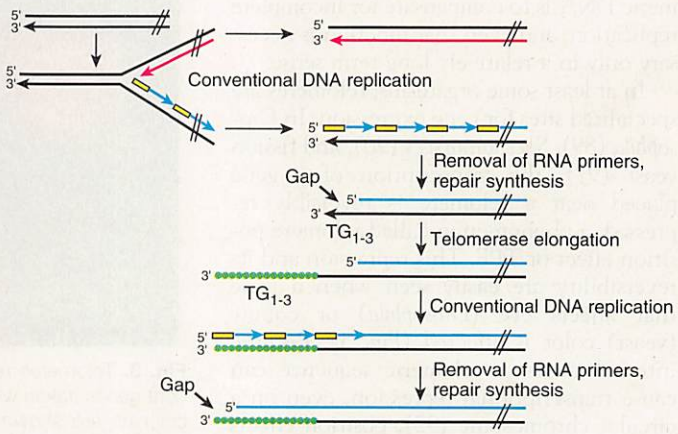

Fig. 2. Replication of the end of a *Saccharomyces* chromosome. Replication of one end of a chromosome is shown. The parental strands are in black; the newly synthesized strands generated by a standard DNA polymerase are in pink or blue. The top or leading strand can be replicated to its very end by a conventional DNA polymerase, represented by the pink arrow. The lower strand DNA, which runs 5′ to 3′ from the center to the end of the DNA molecule, is the $TG_{1\text{-}3}$ strand. The $TG_{1\text{-}3}$ strand must be copied discontinuously, represented in blue. Discontinuous DNA synthesis is RNA-primed: the 8- to 12-base-long RNA primers are represented by yellow boxes. After removal of the RNA primers, the internal gaps can be repaired by a conventional DNA polymerase. However, an 8- to 12-base gap is left at the end of the discontinuously synthesized strand. This gap leaves an 8- to 12-base G tail that can be extended by telomerase (where telomerase-synthesized $TG_{1\text{-}3}$ DNA is in green). A conventional DNA polymerase can synthesize the complementary $C_{1\text{-}3}A$ strand, but again a short gap will be left after removal of the terminal RNA primer. There is no information on the identity of the polymerase that generates the $C_{1\text{-}3}A$ complement of the telomerase-generated DNA.

explain the essential function of *RAP1*. Cells carrying alleles that generate Rap1p with reduced DNA binding activity in vitro have shorter telomeres in vivo (*60*, *83*), which suggests that Rap1p prevents degradation of telomeric DNA.

A yeast chromosome without a telomere causes a transient cell division arrest. This arrest is mediated by the *RAD9* checkpoint (*80*), which detects damaged DNA (*84*, *85*). Thus, another critical function of yeast telomeres is to help the cell distinguish intact from broken chromosomes. Telosomal proteins like those from *Oxytricha*, whose binding is terminus-limited (*67*), provide a biochemical explanation for the cell's ability to distinguish a true telomere from a broken end or an internal stretch of telomeric DNA.

Although a dispensable chromosome without a telomere causes a prolonged cell cycle arrest, a yeast cell with a single chromosome lacking a telomere ultimately resumes division without any loss of viability, even if the broken chromosome is not repaired (*80*). In these cells, the chromosome without a telomere is transcribed, replicated, and segregated for as many as 10 cell divisions before its loss. Thus, at least in yeast, telomeres serve no function that must be carried out in cis in every cell cycle. Moreover, *Drosophila* chromosomes that totally lack telomeric DNA have been isolated and maintained in fly stocks for over a decade (*86*, *87*). *Drosophila* chromosomes without telomeric DNA shorten at the very slow rate expected for incomplete replication (*86*, *88*), and therefore they are not degraded nor do they appear to cause a cell cycle arrest. Thus, these functions of yeast telomeric DNA are probably not universal. Indeed, the only essential function of *Drosophila* telomeric DNA is to compensate for incomplete replication, and even that function is necessary only in a relatively long term sense.

In at least some organisms, telomeres are specialized sites for gene expression. In *Drosophila* (*89*), *Saccharomyces* (*90*), and fission yeast (*91*), the transcription of a gene placed near a telomere is reversibly repressed, a phenomenon called telomere position effect or TPE. This repression and its reversibility are easily seen when a gene that affects eye (*Drosophila*) or colony (yeast) color is affected (Fig. 3). Because internal tracts of telomeric sequence can cause transcriptional repression, even on a circular chromosome (*92*), position effects on transcription must not require any novel aspect of telomere structure, such as a free, single-strand G tail. TPE can be eliminated in cis at a single telomere without affecting chromosome loss (*48*), which demonstrates that the structural requirements for TPE are separable from those required for chromosome stability. Because many genes required for TPE are nonessential (*93*), the ability of the telomere to affect transcription is a dispensable function of yeast telomeres.

Evidence from a variety of organisms suggests that mitotic telomeres often associate both with each other and with the nuclear periphery (*94*–*97*). Such associations are widespread, perhaps ubiquitous, during early stages of meiosis, which suggests that these interactions may be important for homolog pairing, recombination, or some other aspect of meiotic chromosome behavior. In mitotic yeast cells, telomere clustering and a localization at the nuclear periphery may be prerequisites for TPE (*98*, *99*).

Mechanisms for the Maintenance of DNA Ends

Biochemical considerations led to the realization that replication of the very ends of linear DNA molecules requires a different mechanism than replication of the rest of the genome (*79*) (Fig. 2). Different solutions to this dilemma are seen in viruses, plasmids, and organelle DNA (reviewed in *100*). One evolutionarily widespread solution is protein-nucleotide priming of replication, which generates a linear molecule covalently bound to protein at a 5′ terminal nucleotide, not templated by the genome (for example, as in ϕ29 and adenoviruses). Another solution is to have a sequence at the end of the molecule that allows formation of a hairpin, concatamer, or circle that eliminates ends at the time of replication (for example, in vaccinia, T7, and lambdoid viruses). Alternatively, the ends of a linear DNA molecule can be maintained by recombination, as in T4 bacteriophage (*101*) or *Tetrahymena* mitochondrial DNA (*102*).

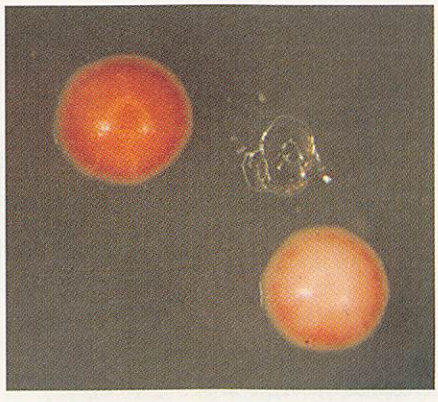

Fig. 3. Telomeres repress transcription of adjacent genes (taken with permission from *74*). Two colonies are shown from a strain in which the *ADE2* gene is next to the right telomere of chromosome V in *Saccharomyces*. About half of the cells produce red colonies, the Ade2$^-$ phenotype, and half produce white colonies, the Ade2$^+$ phenotype. Because the colonies are mostly red or mostly white, both the "off" and "on" transcriptional states are stable. Sectors of opposite color are seen within both red and white colonies, which demonstrates that both states are reversible.

Several different mechanisms also exist to maintain the ends of eukaryotic chromosomes. The termini of *Drosophila* chromosomes usually bear middle repetitive elements that transpose to chromosome ends (reviewed in *103*). Although these terminal transposons are slowly lost by incomplete replication (*104*), their rate of transposition is sufficient to compensate for this loss, and their presence protects internal, single-copy DNA from erosion.

The lengthening of *Drosophila* chromosome ends by transposition is a relatively rare event (*104*). Likewise, for all organisms telomere replication need only be frequent enough to compensate for the very slow loss of DNA that results from incomplete replication. In contrast to the regulated, once-per–cell cycle replication required for the rest of the chromosome, telomere replication could be accomplished as a repair function and need not a priori be regulated by the cell cycle.

In most organisms, telomere replication involves a specialized reverse transcriptase called telomerase. Telomerase is a ribonucleoprotein, its activity depending on both RNA and protein components (*105*). Telomerase circumvents the problem of end replication by using RNA, not DNA, to template the synthesis of telomeric DNA. Before telomerase was discovered, work with ciliates and yeast suggested that telomeric DNA was not templated in a conventional manner. During the formation of the ciliate macronucleus, new telomeres are generated at sites that were previously internal on the chromosome (*4*). Virtually all of these sites have no telomere-like DNA to serve as a template for telomere addition. In contrast, during transformation, yeast telomeres are added to the ends of a linear plasmid, if and only if the end has at least a short stretch of telomeric or telomere-like DNA (*106*). In the yeast transformation assay, *Tetrahymena* and *Oxytricha* telomeres are suitable substrates for telomere addition (*107*–*109*). However, yeast adds its own telomeric sequence to these foreign DNAs, which demonstrates that the newly synthesized telomere is not templated by the preexisting sequence (*12*, *109*, *110*).

Telomerase was first detected in extracts prepared from *Tetrahymena* cells at a stage in macronuclear development when many new telomeres are formed (*111*). Telomerase was discovered by its ability to extend a G strand telomeric oligonucleotide in the absence of a DNA template. Like the activity responsible for forming telomeres during transformation in yeast, *Tetrahymena* telomerase will extend different telomeric G strand oligonucleotides, but the sequence added by the *Tetrahymena* telomerase al-

ways consists of *Tetrahymena* T_2G_4 telomeric repeats, not the sequence used as a primer (*111*). In vitro, *Tetrahymena* telomerase is extremely processive, adding many residues (an average of ~500 bases) to the G strand primer before the enzyme disassociates (*112*).

Telomerase was later identified in extracts prepared from other ciliated protozoans (*113, 114*), humans (*115*), mice (*116*), *Xenopus* (*117*), and very recently from *Saccharomyces* (*118–120*). In each case, telomerase extends the G strand of telomeric DNA. Thus, the expected product of telomerase-mediated replication is a duplex molecule with a single-strand G tail (Fig. 2). A conventional DNA polymerase could theoretically complete telomere replication by synthesizing the complementary C strand (Fig. 2). C strand synthesis by a conventional DNA polymerase that uses an RNA primer will generate a largely duplex molecule with an 8- to 12-base gap at its 5′ end; that is, this molecule will have an 8- to 12-base G tail. Thus, the end product of telomerase replication is also a suitable substrate for telomerase. As long as telomerase adds, on average, more telomeric DNA than that lost by removal of the RNA primer, there will be a net gain of telomeric DNA and the substrate for the next round of telomerase replication will be generated. Whether telomeres grow, shrink, or hover about an average length will depend on a combination of factors, including the telomerase's processivity, its frequency of action at individual telomeres, and the rate of degradation of telomeric DNA.

Genes encoding telomerase RNA have been cloned from ciliates (*121–124*), yeasts (*125, 126*), humans (*127*), and mice (*128*). The RNAs encoded by these genes contain an 8- to 30-base stretch with 1.2 to 1.9 copies of the C strand form of the telomeric repeat of the particular organism from which it was isolated. In *Tetrahymena* (*129*), yeasts, (*125, 126*), humans (*127*), and mice (*128*), the sequence of this region was altered, and the mutant genes were introduced back into the cells. In each case, the altered sequence was either incorporated into telomeric DNA in vivo (*125, 126*) or into telomerase products in vitro (*127, 128*), which indicates that telomerase RNA determines the sequence of telomeric DNA. When the single-copy telomerase RNA gene is deleted in *Saccharomyces* or *K. lactis*, telomeres shorten at exactly the rate expected for incomplete replication (*125, 126*). Thus, in these organisms telomerase must be the major pathway for telomere maintenance. The discovery of telomerase in an organism like *Saccharomyces*, with a heterogeneous telomeric repeat, and in *K. lactis*, with a 25-bp repeat, demonstrates that a telomerase replication mechanism can account for virtually all of the known telomeric DNA sequences.

The protein components of telomerase have been much more elusive than telomerase RNAs. Deletion of the *Saccharomyces EST1* gene, which encodes a 77-kD polypeptide (*130*), yields a phenotype indistinguishable from that of cells lacking telomerase RNA (*125*). Thus, Est1p is required for telomere length maintenance in vivo (*130*). Est1p is specifically associated with telomerase RNA in cell-free extracts (*119*). Moreover, a processive telomerase activity detected by a polymerase chain reaction assay in *Saccharomyces* cell-free extracts is Est1p-dependent (*119*). In contrast, a nonprocessive telomerase detected by a conventional primer extension assay in fractionated *Saccharomyces* extracts is Est1p-independent (*118*). These data can be reconciled if Est1p makes yeast telomerase more processive either by acting directly as a processivity factor or by inactivating a telomerase inhibitor. The genes encoding an 80-kD and a 95-kD protein that co-purify with *Tetrahymena* telomerase activity and telomerase RNA have been isolated (*131*). The 80-kD protein binds specifically to telomerase RNA, whereas the 95-kD protein can be cross-linked to G strand primers. Like Est1p, neither of the *Tetrahymena* proteins has appreciable sequence similarity to RNA or DNA polymerases nor to any known replication accessory proteins, and as yet, neither protein has been shown to be essential for telomerase activity in vivo or in vitro.

Even organisms that normally rely on a telomerase mechanism of replication have alternative pathways for telomere maintenance. Telomeres in *Saccharomyces* strains that lack either *TLC1*, the gene encoding telomerase RNA (*125*), or *EST1* (*130*) slowly shorten. After ~50 to 100 divisions, most of the cells in these cultures stop dividing (*125, 130*). Failure to divide might be a consequence of a *RAD9* arrest more prolonged than that seen when a single telomere is lost (*80*). Alternatively, once telomeric DNA and hence telomere function is gone, degradation may eliminate essential genes, resulting in cell death. Although most *tlc1*Δ and *est1*Δ cells stop dividing, survivors appear spontaneously in all such cultures (*125, 132*). Most survivors have very short telomeres but in addition acquire multiple copies of Y′ or deletion derivatives of Y′ immediately internal to the $C_{1\text{-}3}A/TG_{1\text{-}3}$ terminal repeats (*132*). These tandem Y′ elements, like the *Drosophila* telomeric transposons, might serve as a passive buffer to protect internal sequences from loss. The generation of these survivors requires *RAD52*, a gene needed for most mitotic recombination events. Thus, Y′ acquisition occurs by recombination, not by transposition (*132*).

A *RAD52*-independent recombination process that could theoretically contribute to telomere maintenance has also been detected in *Saccharomyces* (*106*). In contrast to Y′-Y′ recombination, this recombination occurs between two terminal tracts of telomeric repeats. Telomere-telomere recombination proceeds by gene conversion and results in a net increase in telomeric DNA (*133*). It is not known if telomere-telomere recombination contributes to telomere maintenance in mutant or wild-type cells nor if similar pathways can be activated in other organisms. However, in some human cell lines, immortalization and telomere length increases occur even in the absence of detectable telomerase activity (*134–136*). These data suggest that telomerase-independent mechanisms for telomere maintenance also exist in higher cells.

Regulation of Telomere Replication

There are many intriguing aspects of telomere biology that deserve further attention. Of considerable interest is the regulation of telomere replication. In *Saccharomyces*, generation of the single-strand $TG_{1\text{-}3}$ tail is controlled by the cell cycle. The $TG_{1\text{-}3}$ tails are detectable at the very end of the S phase but disappear before the next cell cycle (*39, 40*). Although there is no inherent reason for telomere replication itself to be regulated by the cell cycle, the substrate or product of telomere replication might serve a cell cycle function. Any proposed cell cycle function for telomeres must be reconciled with the fact that a cell with a chromosome lacking one telomere is viable (*80*). One role of telomeres that is consistent with the viability of cells that have a broken chromosome is their function in a cell cycle checkpoint, such as the *RAD9* checkpoint, which is dispensable in the absence of DNA damage (*85, 137*). For example, the transient single-strand G tails that are intermediates in telomere replication may allow the formation of inter- or intratelomeric non–Watson-Crick base-paired structures or may provide a substrate for a terminus-binding protein. Either the DNA structures or the protein-DNA complexes could serve as signals to the *RAD9* cell cycle checkpoint that chromosome replication is complete (*39*).

In addition to the cell cycle regulation of telomere replication seen in yeast, mammalian telomerase is developmentally regulated (*138*). In humans, telomerase activity is not found in most somatic tissues (*139*). In contrast, telomerase is detected in many human tumors (*139*). These data have led to the hypothesis that telomere length may serve as a mitotic clock, which acts to limit the replication potential of human cells both in vivo and in vitro (*52, 140*). The

expression of telomerase in tumor cells may provide a selective advantage that allows these cells to divide indefinitely (*141*).

Telomerase activity and telomere length could be and almost surely are controlled at many levels. Tissue- or stage-specific expression of an essential telomerase subunit is an obvious possibility. However, telomerase RNA is detected in many human somatic cells that lack detectable telomerase activity, which suggests that control of its synthesis is unlikely to be a general mechanism for modulating telomerase activity (*127*). Alternatively, the processivity of telomerase might be regulated. In *Tetrahymena* and in humans, telomerase activity in vitro is very processive, whereas in mice and *Xenopus*, the activity adds only a few bases at a time to G strand primers (*116*, *117*). Because mouse telomeres are exceptionally long (*44*), in vivo there may be accessory factors that increase the processivity of mouse telomerase. In *Saccharomyces*, the Pif1p helicase reduces telomere length and de novo telomere formation in vivo (*142*). Thus, Pif1p acts as if it inhibits telomere replication, although it is not known if this inhibition is on telomerase or on telomerase-independent pathway of telomere maintenance, such as telomere-telomere recombination. The 5′ to 3′ helicase activity of Pif1p could reduce telomerase processivity by dissociating telomerase RNA from its G strand substrate, or it could inhibit telomere-telomere recombination by dissociating recombination intermediates.

Telomerase might also be regulated by telomere accessibility. For example, several Rap1p-interacting proteins in *Saccharomyces* appear to limit telomere replication because depletion of these proteins results in telomere lengthening (*60*, *74*, *143*). Alternatively, DNA structure could affect telomere replication. When G strands assume a G quartet, this structure inhibits their ability to serve as primers for telomerase as well as reduces the extent of telomerase elongation (*144*). Hence, the formation of G quartets might affect both telomere accessibility to telomerase and telomerase processivity. Accessibility to nucleases will also influence telomere length: for example, telomeres in human cells lacking telomerase shorten at a rate ~10 times faster than that expected from incomplete replication (*145*, *146*); this result suggests that in these cells telomeric DNA is degraded, as well as incompletely replicated. Unexpectedly and still inexplicably, overexpression of truncated forms of the *Saccharomyces* telomerase RNA gene causes telomere shortening and loss of telomere position effect (*125*). These results indicate that there is considerable interplay between telomere structure, function, and replication.

In *Saccharomyces*, a surprisingly large number of proteins and conditions affect telomeres. Moreover, even in yeasts and in ciliates our understanding of telomere biology is relatively modest, with new information still emerging at a rapid rate. Telomerase is often proposed as an ideal target for cancer therapy because its activity is widespread among human tumors but absent from most somatic cells. A detailed understanding of telomere replication, including information on telomerase-independent mechanisms of telomere maintenance, will be crucial if telomerase is to become a realistic target for cancer therapy.

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Equality for X Chromosomes

Richard L. Kelley and Mitzi I. Kuroda

In many species, females possess two X chromosomes and males have one X chromosome. This difference is critical for the initial determination of sex. However, the X encodes many functions required equally in males and females; thus, X chromosome expression must be adjusted to compensate for the difference in dosage between the sexes. Distinct dosage compensation mechanisms have evolved in different species. A common theme in the *Drosophila melanogaster* and *Caenorhabditis elegans* systems is that a subtle alteration of chromatin structure may impose this modest, but vital adjustment of the X chromosome transcription level.

Dosage Compensation in *Drosophila*

The predominant dosage compensation mechanism in *Drosophila melanogaster* is hypertranscription of the single male X chromosome in order to achieve an activity equal to that of both female X's (reviewed in *1*). Genetic analyses have identified four genes that are required exclusively for male viability and whose products mediate hypertranscription of the male X chromosome. These genes—*male specific lethal-1*, *-2*, *-3*, and *maleless* (*msl-1*, *-2*, *-3*, and *mle*, collectively called the *msls*)—have been characterized at the molecular level. Antibodies to any one of the MSL proteins specifically recognize hundreds of sites along the male polytene X chromosome (*2–7*). The *msl* mutants display similar phenotypes, and the proteins colocalize on the X chromosome, suggesting that they act in a heteromeric complex. Furthermore, each of the MSL proteins must be functional in order to observe the wild-type chromatin-binding pattern of the remaining three (reviewed in *1*). Direct evidence for a physical interaction between the MSLs has been demonstrated by coimmunoprecipitation of MSL-1 and MSL-2 (*6*).

The biochemical function of the putative MSL protein complex is not understood, but it may function in histone modification. Male X chromatin is highly enriched for an isoform of histone H4 monoacetylated at lysine-16 (H4Ac16) (*8*). Mutation of the corresponding lysine of yeast histone H4 produces altered transcription of several genes (reviewed in *9*). Although the mechanism of altered gene expression is not understood, neutralizing a key positive charge on the NH_2-terminal histone H4 tail may allow greater access of the transcriptional machinery to DNA (*9*). The MSL banding pattern is highly similar to that of H4Ac16, and mutation in any of the *msl* genes prevents accumulation of H4Ac16 on the male X chromosome (*10*). Perhaps one component of the MSL complex is a histone acetyltransferase or an inhibitor of a histone deacetylase.

Sequence analysis shows that MSL-1 and -3 are unlike any previously reported protein (*3*, *4*); MSL-2 contains a zinc-binding motif called the RING finger (*5–7*), and MLE is closely related to human RNA helicase A (*11*). The finding that MSL-2 contains a RING finger present in several other chromatin-binding proteins has led to the suggestion that this subunit provides the recognition specificity to distinguish the X chromosome from the autosomes (*5–7*). Mutations in the RING finger destroy *msl-2* activity (*5*), but so far no RING finger protein has been shown to possess sequence-specific DNA-binding activity (*12*). Two members of this family from *Drosophila* are *Posterior sex combs (Psc)* and *suppressor of zeste 2 [su(z)2]*, which both function in the maintenance of repressive chromatin structure in discrete regions of the genome (*13*).

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One of the more perplexing discoveries is that the MLE protein is likely to act on an RNA substrate. Not only is MLE highly similar to an RNA helicase over its entire length (*11*), but it contains two domains near its NH_2-terminus thought to bind double-stranded RNA (*14*). The glycine-rich heptad repeat near the COOH-terminus may provide another RNA-binding domain. Furthermore, ribonuclease treatment strips MLE protein from polytene chromosomes, whereas it leaves MSL-1, -2, and RNA polymerase bound (*15*). The identity of the RNA substrate or substrates of MLE is not known.

An alternative model for dosage compensation in *Drosophila* is that the autosomal rather than the X-linked genes are subject to differential expression in males and females (*16*). The most compelling argument against this idea is that the MSL proteins display male-specific binding to hundreds of sites along the X, but to very few sites on the autosomes.

Sex-Specific Regulation Through 3′ and 5′ Untranslated Regions

The X to autosome ratio (X:A) controls *Sex lethal* (*Sxl*), the master regulator of both sexually dimorphic development and dosage compensation (reviewed in *17*). Females (X:A = 1.0) make SXL protein, which represses the *msl* pathway and results in basal transcription of both X chromosomes. Males (X:A = 0.5) lack SXL protein, leaving the *msl* pathway active for hypertranscription of most X-linked genes. SXL is an RNA-binding protein that regulates alternative splicing of target transcripts in the sex determination pathway (*18*, *19*).

Characterization of the *msl-2* gene has illuminated how *Sxl* may restrict MSL function to males. Although *mle*, *msl-1*, and *msl-3* produce the same transcripts in both sexes, *msl-2* RNA is alternatively spliced in males and females (*5*). A small intron is removed from the 5′ untranslated region (UTR) of male transcripts but retained in female transcripts. SXL protein is likely to directly control alternative splicing of this intron because consensus SXL-binding sequences [poly(U)] are located adjacent to both the 5′ and 3′ splice sites (Fig. 1). Female cells that are mutant for *Sxl* splice the 5′ intron (*7*) and derepress *msl-2* translation (*6*), further supporting the idea that *msl-2* is a direct target of *Sxl*.

The two other well-characterized targets of *Sxl* regulation, *Sxl* itself and *tra*, are spliced in females to generate productive open reading frames (Fig. 1). The default male splicing pattern introduces a premature stop codon in both transcripts (*18*, *19*). The sex-specific alternative splice of *msl-2* RNA does not affect the open reading frame, yet MSL-2 protein is readily detectable in males but absent in females (*5*–*7*). It is unclear how translation of the female *msl-2* RNA is blocked. There are additional potential SXL-binding sites in the 3′ UTR, deletion of which causes partial derepression of *msl-2* in females (*7*). Similar sites are found in the 3′ UTR of some *msl-1* transcripts and may also contribute to its down-regulation in females (*3*, *20*). The presence of SXL-binding sites in both the 5′ and 3′ UTRs of female *msl-2* transcripts suggests that translation might be directly influenced by SXL protein sequestering the target RNA in the nucleus or preventing association with ribosomes. Alternatively, SXL may indirectly affect translation by blocking the removal of a negative regulatory element located in the 5′ intron.

Ectopic expression of MSL-2 protein in otherwise wild-type females is sufficient to assemble all of the known dosage compensation components onto both female X chromosomes and result in the more diffuse X chromatin structure normally found only in males (*6*). Such females suffer decreased viability, and surviving females show delayed development and poor fertility. The fact that dosage compensation components can assemble on female X chromosomes when only MSL-2 is abnormally expressed further supports the idea that *msl-2* is the primary target of *Sxl* repression in females. Whereas the other MSL proteins are synthesized in wild-type females, they are either unstable or unable to assemble onto the X chromosomes in the absence of MSL-2 (*4*, *6*, *20*, *21*).

A Second Dosage Compensation Pathway May Operate in Females

Several observations indicate that flies use a second mode of dosage compensation. *Sxl* mutant female embryos die, at least in part because the *msl* pathway is derepressed, but *Sxl*;*msl* double mutants still die (*22*, *23*). A second pathway controlled by *Sxl*, but independent of the *msl* genes, would explain this result (*24*). In support of this model, *runt*, an X-linked gene expressed early in embryogenesis, is regulated by an *Sxl*-dependent, but *msl*-independent, mechanism (*25*). Hence, *Sxl* must either directly or indirectly control a second dosage compensation pathway that is active soon after zygotic transcription begins.

The finding that SXL protein might regulate the translation of *msl-2* (and possibly *msl-1*) has prompted consideration of a direct dosage compensation model, in which SXL down-regulates translation or stability of X-encoded RNAs in females without affecting splicing. Inspection of the *runt* sequence showed that it also contains a cluster of three potential SXL-binding sites in its 3′ UTR. A further search of the *Drosophila* sequence database for transcripts with clusters of SXL-binding sites (U_8 or AU_7) in their 3′ UTRs revealed that most genes with such an organization map to the X chromosome (*6*). The exceptions support the rule, as the only known autosomal genes with clustered SXL-binding sites in their 3′ UTRs are *msl-1* and *msl-2*.

The hypothetical direct *Sxl* pathway differs from the *msl* dosage compensation pathway in several critical respects. The *msl* mechanism increases transcription in males, possibly by altering chromatin structure, whereas the direct *Sxl* pathway reduces translation or RNA stability in females. It is thought that the two dosage compensation pathways act in parallel during development (*26*, *27*). Hence, the *Drosophila* X chromosome may be interspersed with many genes that are up-regulated in males, some that are down-regulated in females, and a few that are not dosage compensated.

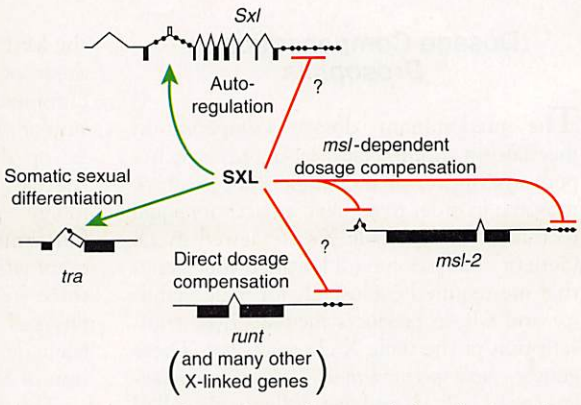

Fig. 1. Functions of the *Drosophila* SXL protein. SXL regulates somatic sexual differentiation in females by controlling the splicing of *tra* RNA (*19*). SXL is required for maintenance of the female state by positively autoregulating its own expression at the level of RNA splicing (*18*). The presence of many copies of poly(U) in the 3′ UTRs of some *Sxl* transcripts raises the possibility that SXL might also negatively regulate its own translation (*6*). SXL may repress *msl*-dependent dosage compensation in females by binding to poly(U) clusters in the untranslated regions of *msl-2* RNA (*5*–*7*). SXL may directly control a second dosage compensation pathway that acts in females to reduce expression of a subset of X-encoded transcripts, such as *runt*, that contain poly(U) clusters in their 3′ UTRs (*6*). The target transcripts are schematically shown with noncoding regions as horizontal lines, coding exons as filled boxes, exons with stop codons as open boxes, introns as bent lines, and poly(U) sites as filled circles. Green arrows indicate a positive regulatory function, and red bars indicate a postulated negative regulatory role. *Sxl* also plays a role in female germline development.

Dosage Compensation in *Caenorhabditis elegans*

In *C. elegans*, XX animals are hermaphrodites and XO animals are males. In contrast to *Drosophila*, transcription of the male X is the basal state, and transcription of both X chromosomes in the hermaphrodite is reduced by approximately 50% to achieve this level (reviewed in *28*). This strategy for dosage compensation is complementary to the *msl* pathway in *Drosophila*, but faces the same conceptual difficulty: How may a twofold difference in gene expression be imposed on the diverse mechanisms controlling the genes on a whole chromosome? As in *Drosophila*, this is achieved by the action of one or more X chromosome–associated proteins that act in a sex-specific manner (*29*).

The genes required in hermaphrodites can be divided into two groups (Fig. 2): (i) genes that affect both sex determination and dosage compensation (*sdc* genes), and (ii) genes that affect only dosage compensation (*dpy* or *dumpy* genes). All of these—*sdc-1*, *sdc-2*, *sdc-3*, *dpy-21*, *dpy-26*, *dpy-27*, *dpy-28*, and *dpy-30*—are required for reduction of X-linked gene expression in hermaphrodites (reviewed in *28*). In general, loss of both maternal and zygotic contributions of wild-type *sdc* or *dpy* functions results in XX inviability. Escapers, or animals lacking only the zygotic functions of these genes, have a dumpy phenotype: They have short, fat bodies and are egg-laying defective. Notable exceptions are *sdc-2*, which requires zygotic function for viability (*30*), and *sdc-1* and *dpy-21*, which display weaker effects on viability than the others (*31*, *32*).

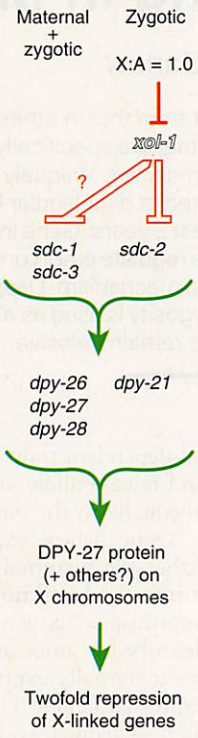

Fig. 2. The *C. elegans* dosage compensation regulatory hierarchy drawn from the perspective of the XX hermaphrodite. Genes on the left have both maternal and zygotic components, and genes on the right function zygotically. The X:A ratio is the primary determinant of sex. When the ratio is 1.0, the earliest known gene in the pathway, *xol-1*, is repressed. This results in zygotic activation of *sdc-2* and function of all the *sdc* and *dpy* gene products to repress X-linked transcription twofold. It is not yet known which components may directly associate with the X, like DPY-27, and which products regulate this event. The *sdc* genes are placed upstream in the hierarchy based on their additional role in sex determination. *dpy-30* is not placed in the hierarchy because its position is currently under reevaluation (*49*). Green arrows represent positive interactions and red bars depict negative regulation. The open red bars indicate that *xol-1* repression of the *sdc* genes does not occur in the hermaphrodite.

The simplest position for the *sdc* genes in the genetic hierarchy is in a common regulatory pathway leading to independent sex determination and dosage compensation branches. *sdc-1* encodes a 139-kD protein with seven TFIIIA-like zinc finger motifs, suggesting that it functions in gene regulation (*33*). *sdc-2* (*34*) may be a direct target of the first known gene in the hierarchy, *xol-1* (*35*). SDC-3 regulates sex determination and dosage compensation through independent domains (*36*, *37*). Sex determination–specific mutations affect an apparent adenosine triphosphate (ATP)–binding domain, whereas dosage compensation–specific mutations affect a pair of zinc finger motifs. The presence of putative DNA-binding motifs in both SDC-1 and SDC-3 is compatible with a positive regulatory effect on the *dpy* genes (Fig. 2). Alternatively, one or more of the SDC proteins could participate more directly in repression of the X chromosome.

The dosage compensation–specific DPY proteins are most likely direct regulators of the X chromosome. This is the case for DPY-27, which is associated with the X chromosomes in hermaphrodites but not in males, starting at the 30-cell stage of embryogenesis (*29*). The association of DPY-27 protein with the X chromosomes in hermaphrodites provides strong evidence that the prevailing model for *C. elegans* dosage compensation is correct; the mechanism operates by decreasing gene expression in hermaphrodites rather than increasing gene expression in males. Furthermore, the DPY-27 sequence provides support for a model in which dosage compensation is achieved through regulation of higher order chromosome structure (*29*). DPY-27 is a member of the SMC family of proteins (reviewed in *38*), which are involved in several aspects of chromosome dynamics, including chromosome condensation in *Xenopus* extracts (*39*) and chromosome segregation in budding and fission yeast (*40*). Members of this family are characterized by an NH_2-terminal ATP-binding motif, a conserved COOH-terminal domain, and a central coiled-coil region reminiscent of motor proteins such as myosin or kinesin. The ATP-binding motif in DPY-27 is essential for its function (*29*), consistent with a model in which regulation of chromosome structure by SMC proteins is energy dependent (*38*). The homology to proteins required for general chromosome segregation of yeast fits well with a previously established link between chromosome behavior and dosage compensation: *dpy-26* and *dpy-28* mutants (but not *dpy-27*) have a nondisjunction phenotype (*32*, *41*). DPY-27 protein, and perhaps other factors required for X chromosome dosage compensation in *C. elegans*, may be specialized versions of proteins that regulate general chromosome function in many organisms. Some part of *dpy-30* function is indeed general, as mutations affect the development and morphology of both sexes (*42*). Consistent with this phenotype, *dpy-30* encodes a 123–amino acid nuclear protein present in both sexes at all stages of development (*43*).

The Sex Specificity of X Chromosome Repression

The *sdc* and *dpy* genes are members of a genetic hierarchy that responds to the X:A ratio in the zygote (Fig. 2) (reviewed in *28*). When the X:A ratio is 0.5 (male), the *xol-1* (XO lethal) gene represses function of the *sdc* and *dpy* genes (*44*). When the X:A ratio is 1.0 (hermaphrodite), *xol-1* is inactive, the *sdc* and *dpy* genes are functional, and dosage compensation occurs. However, most of the *sdc* and *dpy* genes have significant maternal contributions, suggesting that sex specificity is not strictly determined by zygotic activation of these genes in response to the X:A ratio (*28*). In addition, although the DPY-27 protein is associated with the X chromosomes only in hermaphrodites, it is present in the male nucleus, indicating that regulation does not occur at the level of its synthesis (*29*). As with *Drosophila msl* gene products, the continued expression, stability, or localization of maternally supplied dosage compensation regulators might be regulated by a gene with strict zygotic function, analogous to *msl-2*. The best candidate in *C. elegans*, based on its essential zygotic component, is *sdc-2* (Fig. 2) (*30*). Expression of *sdc-2* is thought to be directly repressed by *xol-1* in males, which in turn is zygotically expressed in response to the X:A ratio (*35*).

Sex-specific regulation of *xol-1* promises to shed light on how the X:A ratio is measured, as *xol-1* may be the direct target of the primary sex determination and dosage compensation signal (*35*). At the peak of its expression, *xol-1* RNA is approximately 10 times more abundant in males than in hermaphrodites. An *xol-1–lacZ* translational fusion (*35*) and an analogous transcriptional fusion (*45*) exhibit XO-specific β-galactosidase expression in embryos, suggesting that some component of the X:A ratio acts through the *xol-1* promoter. The identity of X:A numerator and denominator elements and further analysis of the sex-specific regulatory elements of *xol-1* should be extremely informative as to the molecular mechanism for X:A measurement (*35*, *46*).

It is still an open question whether the C.

elegans X chromosome is dosage compensated on a gene by gene basis, as the *Drosophila* X appears to be, or by a long-range chromosomal phenomenon like X inactivation in mammals (reviewed in *1*, *47*). If the mechanism is more similar to X inactivation, then it could spread long distances to neighboring chromatin in X:A translocations. If it operates on a gene by gene basis, then X-linked genes moved to autosomes should retain dosage compensation. Data to support either of these models are scarce, in large part because of the transformation system in *C. elegans*, in which microinjected DNA is usually maintained in extrachromosomal arrays rather than within the genome in single copy. Therefore, individual X-linked genes have not been assayed for dosage compensation when inserted into autosomes (*28*). However, in an unpublished study by Hsu and Meyer (cited in *28*), an autosomal gene, *unc-54*, was dosage compensated when inserted onto the X chromosome, so in this example any hypothetical cis-acting elements must have been able to function over distances of several kilobases. In addition, in one genetic study, mutations in several autosomal loci were not fully complemented by the autosomal portion of a translocation between the X and chromosome V, suggesting that the X signals might act over long distances to repress juxtaposed chromatin (*48*). The ability to stain for DPY-27 protein on translocation chromosomes should begin to allow the resolution of this very fundamental question.

Things to Come . . .

In this review, we have highlighted aspects of X chromosome dosage compensation in two model organisms, fruitflies and nematodes. A third system that is of extreme interest to the chromosome field is the X-inactivation mechanism used by female mammals (*47*). A potentially fundamental breakthrough in that field may be forthcoming, as researchers determine whether inactivation of a whole chromosome relies, at least in part, on the expression of a nonprotein-coding RNA molecule, termed Xist, from the inactive X chromosome. We await with excitement the elucidation of this third, distinctive mechanism of X chromosome dosage compensation.

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Gametic Imprinting in Mammals

Denise P. Barlow

Embryonic development in mammals is distinct from that in other vertebrates because it depends on a small number of imprinted genes that are specifically expressed from either the maternal or paternal genome. Why mammals are uniquely dependent on sexual reproduction and how this dependency is dictated at a molecular level are questions that have been intensively investigated during the past 2 years. Gene inactivation experiments have confirmed predictions that imprinted genes regulate embryonic and placental growth and that DNA methylation is part of the imprinting mechanism. Despite these considerable achievements, the reason why imprinted hemizygosity is used as a mechanism to regulate the intrauterine growth of mammalian embryos remains elusive.

In mammals some genetic traits show parental dependency and are only expressed when inherited from one parent. Two types of parental dependency are currently known. The first has a trivial cause and is due to an unequal distribution of genetic information between male and female gametes. Examples of this type include traits encoded by mitochondrial genes, Y chromosome–linked genes, and maternal-effect genes. The second type, known as gametic or genomic imprinting, is more of an enigma whose role in mammalian development and disease is not yet fully appreciated. Gametic imprinting describes those parental-dependent traits in which both the male and female allele are present but function unequally in the embryo.

Genes whose expression is restricted to either the maternal or paternal allele constitute the best known example of gametic imprinting. Sixteen such genes have been described in mice and humans, 5 of which are maternally expressed and 11 paternally expressed (Table 1). However, other traits such as trinucleotide repeat amplification, host-defense methylation responses, asynchrony of sister chromatid behavior, and meiotic recombination also exhibit parental dependency (*1*). Whether these latter traits arise from gametic imprinting is not yet clear; therefore, they will not be considered further in this brief review.

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Why Are Genes Imprinted?

The existence of any parental-dependent trait in mammals is a puzzle, because the advantage offered by a biparental contribution is clear from the recessive nature of most mutations. What then is the function of the hemizygosity induced by gametic imprinting? Earlier work based on experimentally derived androgenotes (diploid embryos with two paternal genomes) and gynogenotes (diploid embryos with two maternal genomes), and on mice that were diploid but had inherited both copies of individual chromosomes from one parent, showed that imprinted genes are essential in development and may play a growth regulatory role in the embryo and neonate (2). Interestingly, placental development appeared to depend on paternally expressed genes, and embryonic growth on maternally expressed genes, suggesting a link between imprinting and the control of intrauterine embryonic growth. This presumed link has been strengthened by recent analyses of mice harboring inactivated copies of imprinted genes. Of five imprinted genes studied, four caused defects in embryonic or placental growth [*IGF2*, *IGF2R/MPR300*, *H19*, and *MASH2*], and one was lethal before the onset of maximal embryonic growth at midgestation (*WT1*) (Table 1). (Human gene symbols are used throughout text and in Table 1.) However, not all of these genes showed the division of labor suggested by the analysis of androgenotes and gynogenotes. *MASH2*, for example, is a maternally expressed gene essential for formation of the placenta (3). Although it is too soon to conclude that all imprinted genes have a similar function, the significance of these results is emphasized by the fact that of the 263 gene "knockouts" analyzed in mice, 4% affect embryonic growth, 11% are lethal before midgestation, and 85% have no growth defect (4).

The observation that imprinted genes influence embryonic growth in mammals, combined with the apparent absence of imprinted genes in oviparous vertebrates, offers support for the suggestion that the role of gametic imprinting lies in the control of intrauterine embryonic growth. This presumed role is further strengthened by the observation that gametic imprinting has also been observed in the endosperm of a few angiosperm plants, a tissue suggested to be analogous to the mammalian placenta (5). Although the experimental evidence supports such a role for gametic imprinting, the explanation of why imprinted genes fulfill this particular function awaits the examination of a larger number of imprinted genes. From the evidence so far, it is not possible to distinguish between simplistic explanations, such as a role in maintenance of sexual reproduction, and the more exotic explanations, exemplified by the parental conflict hypothesis (5) and the trophoblastic disease protection hypothesis (6).

Table 1. Mammalian genes that show parental-specific expression in certain developmental stages and tissues. P, paternal-specific; M, maternal-specific; R, random; nd, not done; *WT1*, Wilms tumor suppressor (*20*); *INS*, insulin [two mouse loci, one human locus (*22*)]; *IGF2*, insulinlike growth factor type 2 (*21*); *H19*, non–open reading frame (ORF) RNA (*21*); *p57KIP2*, cyclin-dependent kinase inhibitor (*31*); *MASH2*, helix-loop-helix transcription factor (*3*); *SNRPN*, small nuclear riboprotein particle SmN (*21*); *ZNF127*, zinc finger protein (*21*); *PAR1* and *PAR5*, anonymous transcripts in the Prader-Willi consensus region (*27*); *IPW*, non-ORF RNA (*28*); *IGF2R/MPR300*, insulinlike growth factor type 2 receptor (also known as the mannose 6-phosphate 300-kD receptor) (*21*); *MAS*, cell surface receptor, putative oncogene (*29*); *XIST*, X chromosome–inactive specific transcript (*21*); *PEG1/MEST*, mesoderm-specific transcript (*30*); *SP2*, also known as *U2af1rs1*, a protein related to U2 small nuclear ribonucleoprotein auxiliary factor (*21*).

Gene	Expressed allele	Chromosome	
		Mouse	Human
WT1	M	2	11p
INS	P	7	11p
IGF2	P	7	11p
H19	M	7	11p
p57KIP2	M	7	11p
MASH2	M	7	nd
SNRPN	P	7	15q
ZNF127	P	7	15q
PAR1	P	nd	15q
PAR5	P	nd	15q
IPW	P	nd	15q
IGF2R/MPR300	M	17	6q
MAS	P	17	nd
XIST	P/R	X	X
PEG1/MEST	P	6	nd
SP2	P	11	nd

How Are Genes Imprinted?

Imprint acquisition. Gametic imprinting requires that parental alleles be distinguished in every cell of the early embryo. The distinguishing mark, or imprint, that almost certainly arises during gametogenesis or before pronuclear fusion in the zygote (Fig. 1) must have three properties: (i) It must be present in one gamete and be sufficiently stable in mitosis to be propagated to every cell of the embryo. (ii) It must remain restricted to one parental chromosome in diploid cells. (iii) It must be reset in the germ line after the sex of the embryo is determined. Thus, the imprinting process is predicted to have an epigenetic component (the "imprint") that marks one parental chromosome, and a genetic component (the DNA sequence or "imprinting box") that is modified by the imprint.

Although certain DNA binding proteins such as the *Drosophila* Polycomb proteins could fulfill the requirements of the imprint (7), DNA methylation is so far the best candidate. In mammals DNA methylation is restricted to cytosine, usually in a CpG dinucleotide (^{m}CpG). After replication DNA will contain hemimethylated CpG dinucleotides that are the unique substrate for DNA methyltransferase (DNMTase) (8). DNMTase action is thus restricted to previously methylated sequences and acts to stably propagate chromosome-specific methylation patterns to daughter cells. Furthermore, in keeping with the properties required of the imprint, genomic methylation patterns are removed and reset during gametogenesis and in the preimplantation embryo (9). Thus, methylation has all of the hallmarks required of the imprint.

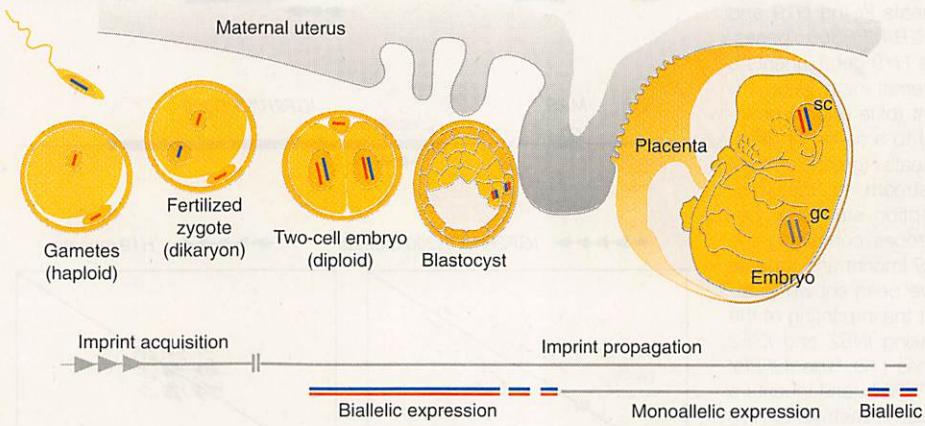

Fig. 1. Gametic imprinting is a multistep process. The key stages of gametic imprinting are shown. Imprint acquisition occurs when the genome is haploid, in the gametes or the dikaryon zygote, but may be modified later in development. In the later embryo, the imprint is stably propagated through mitosis and remains restricted to one parental chromosome. Monoallelic expression of several imprinted genes occurs late in the preimplantation embryo, and can be lost in some tissues of the later embryo or adult. Two types of diploid cell are depicted in the embryo: sc, somatic cell with imprinted chromosomes; and gc, germ cell with nonimprinted chromosomes. The oocyte polar body is illustrated up to the two-cell stage. Paternal expression is indicated by the blue line and maternal expression by the red line.

All imprinted genes so far examined contain DNA sequences methylated in a parental-specific manner (*8*). In some cases this methylation mark is acquired late in embryonic development. However, three imprinted genes (*IGF2R/MPR300*, *H19*, and *XIST*) and one imprinted transgene (RSVIgmyc, a complex mouse genomic fragment with an immunoglobulin–c-*myc* fusion) (*10*) have been shown to inherit a methylation imprint from one gamete. In these four cases the methylation imprint resists preimplantation demethylation, is maintained in somatic tissues, and is reset in the germ line. Interestingly, the DNA sequences carrying these gametic imprints preferentially retain methyl groups (*11*) when DNMTase activity is limiting. The significance of gametic methylation imprints has not yet been directly tested by genetic manipulation. However, a role for these imprints is supported by the finding that *H19* transgenes, containing the imprinted sequence, show the expected monoallelic expression pattern when integrated at independent loci (*12*).

The *IGF2R/MPR300* gene, the *XIST* gene, and the RSVIgmyc transgene are methylated maternally, whereas the *H19* gene is paternally methylated (*10*). DNA sequences carrying imprints from the same parent but not those imprinted by the other parent might be expected to share some common features. But, so far, no direct sequence homology has been found among the three maternally imprinted genes. The DNA sequences carrying both maternal and paternal gametic methylation imprints do, however, have two features in common. First, they resemble CpG islands, containing CG-rich regions of 200 to 1500 base pairs (bp) with a balanced CG:GC ratio (*13*). These imprinted sequences differ from standard CpG islands because they are methylated, albeit only on one parental allele. Because spontaneous deamination of ^{m}C yields a T residue that is not repaired by the cell, methylation is expected to lead to loss of a CpG island (*13*). This may be prevented because the methylation imprint is removed early in the germ line and only reset in mature gametes (*9*); thus, imprinted islands are methylated for a relatively short period in the mammalian life span. The second feature shared by imprinted DNA sequences is that they contain, or are closely associated with, a region rich in direct repeats (Fig. 2). These repeats range in size from 25 to 120 bp, are unique to the respective imprinted regions, but have no obvious homology to each other or to highly repetitive mammalian sequences. The direct repeats may be an important feature of gametic imprinting, as they have been found in all imprinted genes analyzed to date and are also evolutionarily conserved (*14*).

Reading the imprint. Monoallelic expression of imprinted genes is not always coincident with the onset of expression and can also vary in development, differentiation, and disease (Fig. 1). The embryonic genome is activated at the two-cell stage coincident with degradation of maternally stored messenger RNA (*15*). Some imprinted genes such as *XIST* and *H19* (*10*) show monoallelic expression in early preimplantation embryonic stages. However, analyses of preimplantation embryos derived from androgenotes and gynogenotes and of embryonic stem (ES) cells (which resemble blastocyst-stage embryos) show equal parental expression of several imprinted genes. The *IGF2R/MPR300* gene, for example, is expressed in preimplantation androgenetic embryos, and from both parental alleles, in ES cells and in all preimplantation stages up to late blastocyst (*16*). The separation in time between acquisition of the gametic imprint and onset of monoallelic expression, which can occur in blastocysts containing several hundred cells, suggests that the imprint itself does not have to be a primary inactivation event and that the imprinting mechanism may involve additional trans-acting molecules that read the imprint. This suggestion is supported by two examples that have been molecularly characterized. In the first, a methylation imprint appears to directly repress the mouse *H19* gene on the paternal chromosome, and maternal expression of *H19* appears to inhibit *IGF2* and *INS1* expression in cis (*17*), possibly by enhancer competition (Fig. 2). In the second example, biallelic expression of the human *IGF2* gene occurs in neonatal liver through the recruitment of an upstream promoter that appears to be insensitive to the imprint (*18*).

Maintenance of monoallelic expression. The extent of monoallelic expression also varies in development and differentiation and differs between humans and mice. The mouse *IGF2R/MPR300* gene is maternally expressed in every tissue in all tested mouse strains. In contrast, monoallelic expression of the human gene appears to be a polymorphic trait, occurring in all tested tissues but only in a minority of the human population (*19*). A similar polymorphism is shown by the human *WT1* gene in placental tissue (*20*). Biallelic expression of the mouse and human *IGF2* gene occurs in some neural tissues, and in the human, also in adult liver (*18, 21*). Finally, the mouse *INS1* and *INS2* genes only show monoallelic expression in the extraembryonic membranes but not in the pancreas (*22*). In addition to the variation in monoallelic expression that occurs in development and differentiation, *IGF2* has been shown to switch to biallelic expression in tumors and other human diseases (*23*), and mice with an inactive maternal *IGF2R/MPR300* allele can in some circumstances switch on their paternal allele (*16*).

One key experiment has presented strong evidence that monoallelic expression of several imprinted genes depends on DNA methylation. Mice deficient for DNMTase die in the early postimplantation period but advance sufficiently so that gene expression can be analyzed. In the absence of DNMTase, monoallelic expression of four imprinted genes (*IGF2*, *IGF2R/MPR300*, *H19*, and *XIST*) was lost in postimplantation embryos (*11*). Surprisingly, the loss of monoallelic expression occurred, as predict-

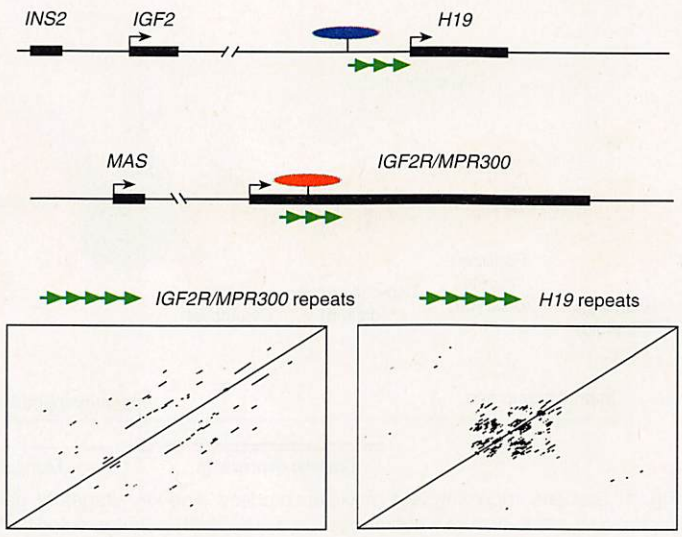

Fig. 2. Gametic methylation imprints and direct repeats in the *H19* and *IGF2R/MPR300* genes. The *H19* gene inherits a paternal methylation imprint (blue ellipse) proximal to a series of direct repeats (green arrows) upstream to the transcription start site. Sequences containing the *H19* imprint and repeats have been shown to affect the imprinting of the flanking *INS2* and *IGF2* genes (*17*). The *IGF2R/MPR300* gene inherits a maternal methylation imprint (red ellipse) directly covering a series of direct repeats (green arrows) that lie in the second intron (*10*). The relative position of the *MAS* gene, which is imprinted only when weakly expressed, is shown (*29*) (the significance of the *IGF2R/MPR300* intronic repeats and of the close linkage to *MAS* has not been analyzed). The boxes at the bottom show the extent of the direct repeats in the *H19* and *IGF2R/MPR300* genes in a 1500-bp region (covered by the green arrows). The boxes were generated by a sequence comparison program that aligns the sequence to itself, as described (*14*).

ed, by the gametic methylation imprints and not, as predicted, by the expected role of methylation in silencing gene expression. The silent, methylated *H19* and *XIST* alleles were activated, whereas the active, methylated *IGF2R/MPR300* allele was silenced. The active *IGF2* allele, which has no obvious gametic imprint, was also silenced. The simplest interpretation of these results is that methylation modulates the access of DNA binding proteins that repress or activate transcription. However, this may be an oversimplification, because in the case of *IGF2*, allele-specific repression most likely results from activation of the upstream *H19* gene (*17*). It remains to be seen if a similar mechanism of enhancer competition could also explain the apparent dependency of *IGF2R/MPR300* expression on methylation.

Because mammalian DNMTase has primarily a maintenance activity, it is unlikely to be involved in the de novo establishment of the gametic imprint. It remains possible that there are other methylating enzymes that carry out this role whose action is maintained by DNMTase in the postimplantation embryo. Alternatively, gamete-specific proteins may convert DNMTase into an imprinting enzyme. The recent characterization of genomic sequences bearing gametic methylation imprints may facilitate the identification of the enzyme responsible for the gametic imprint and, in turn, the identification of as yet unknown imprinted genes.

Future Developments

The 16 imprinted genes listed in Table 1 have been arranged to highlight their clustering into chromosomal domains. Seven imprinted genes [and possibly three more (*PAR1*, *PAR5*, and *IPW*) that have not yet been mapped in the mouse] lie in two separate domains on mouse chromosome 7 (*2*). These two imprinted chromosomal domains are separated in the human onto chromosomes 11 and 15. Genetic experiments in which more than 80% of the mouse genome was analyzed identified only seven chromosomal domains that contained genes expressed in a parental-specific manner in embryonic development (*2*). Because the total number of imprinted genes in mammals is predicted (*24*) to be between 100 to 200, clusters of 15 to 30 imprinted genes would be expected. Although clustering of imprinted genes may predict aspects of the imprinting mechanism, there are two caveats that must be considered. First, the observed clustering may reflect a sampling bias arising from concentrating on regions that flank known imprinted genes (additionally, imprinted expression of flanking genes may also represent nonspecific effects on weak promoters). Second, the number of imprinted domains may have been underestimated if some imprinted genes do not have a major effect on embryonic development. Despite these caveats, it is clear that imprinted genes are clustered. This may imply that groups of genes are imprinted by a common cis-acting sequence, or that other long-range effects are involved.

An analysis of the imprinted clusters on mouse chromosome 7 and human chromosome 15q11-13 (Table 1) has confirmed that a single gene or regulatory element can regulate monoallelic expression of distant imprinted genes in cis (*17*, *25*). The identification of an unusual non–open reading frame–imprinted RNA in both of these clusters, one of which (*H19*) shares structural homology with the *XIST* gene (*14*), adds further support to the concept that imprinted genes are functionally grouped. In addition, the description of other parental-specific effects on chromosomal function, such as asynchrony of sister chromatid behavior and meiotic recombination, which span several megabase pairs of DNA and encompass nonimprinted and imprinted genes (*1*), also indicates that long-range effects are part of the imprinting mechanism. Functional grouping of imprinted genes would predict that genes would not retain imprinted behavior when transferred to other chromosomal sites. However, the *H19* gene and the RSVIgmyc transgene do retain imprinted expression at other chromosomal positions (albeit in a background-dependent manner), whereas the *IGF2* gene showed inconsistent behavior (*10*, *12*, *26*), suggesting that cis-acting or domain effects may not always be dominant. Thus, although the significance of chromosomal clustering of imprinted genes is not yet clear, it may be that gene mapping will offer a shortcut to isolating other imprinted genes.

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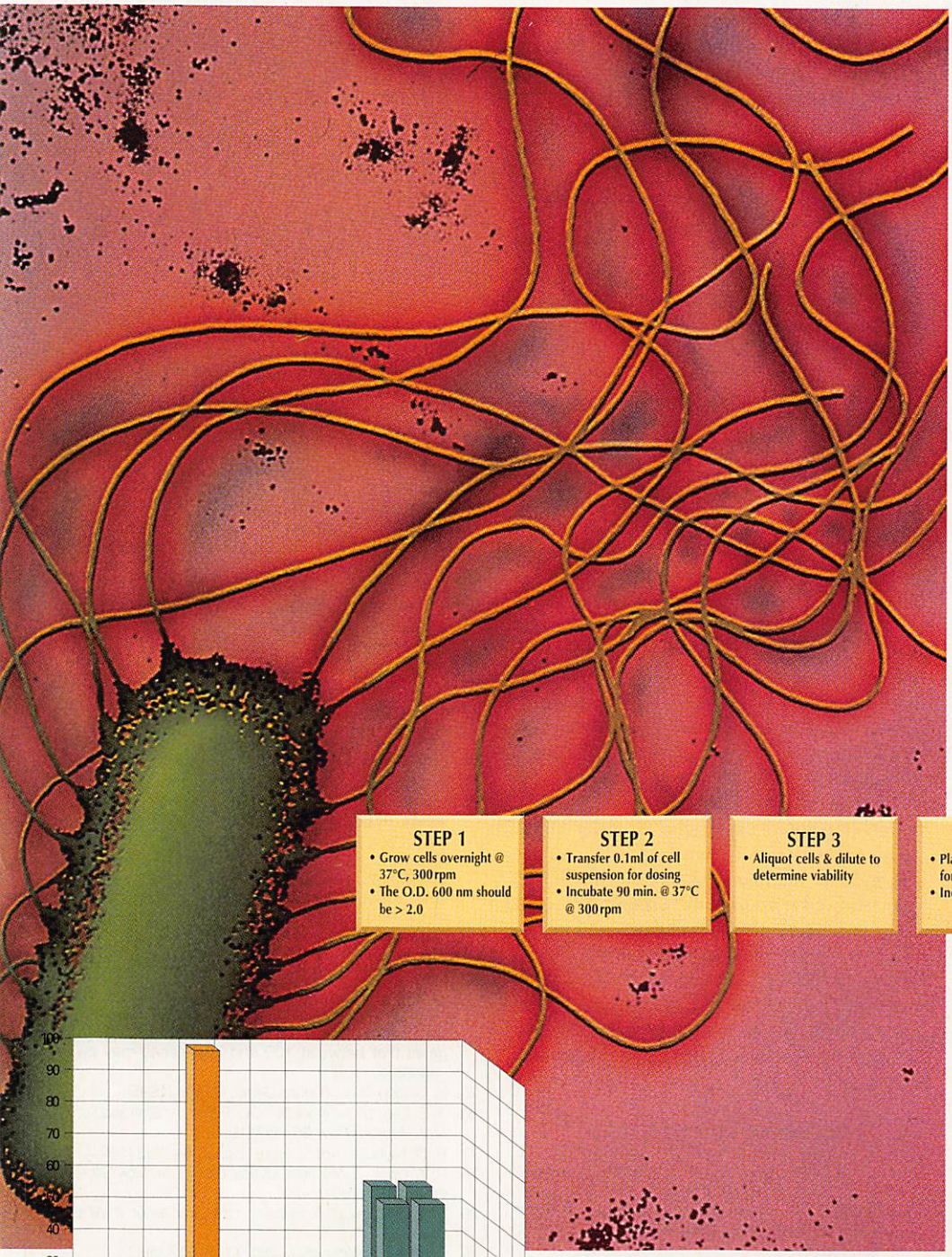

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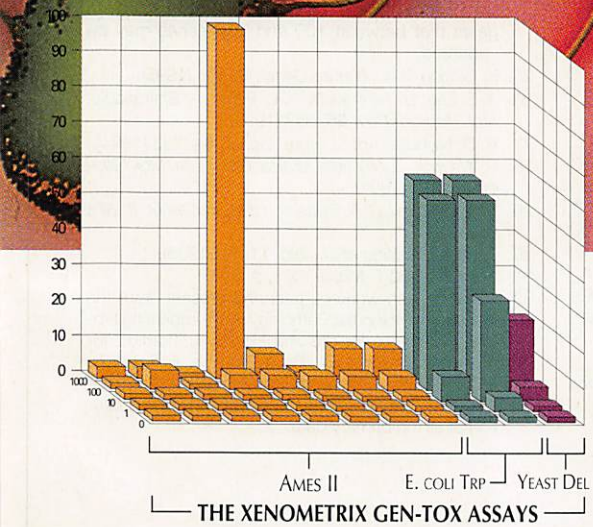

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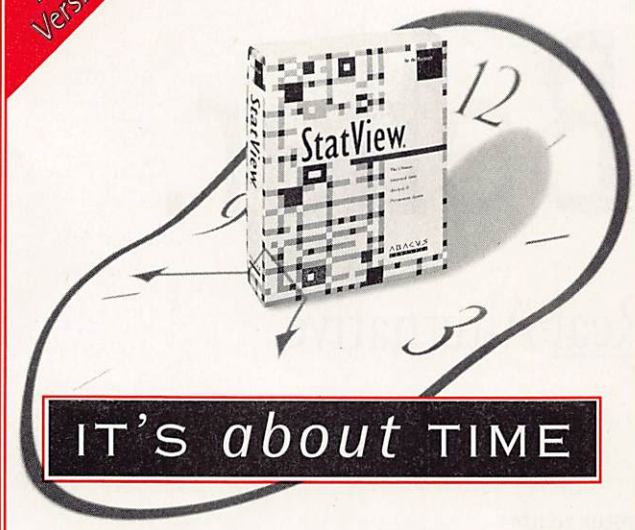

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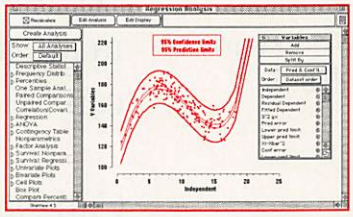

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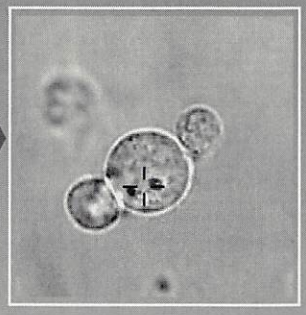

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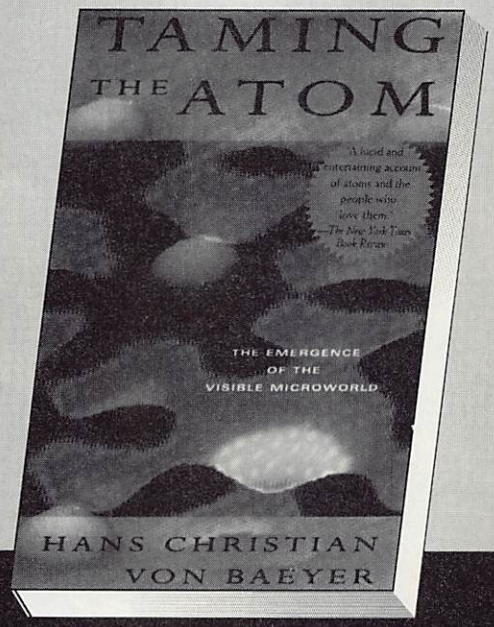
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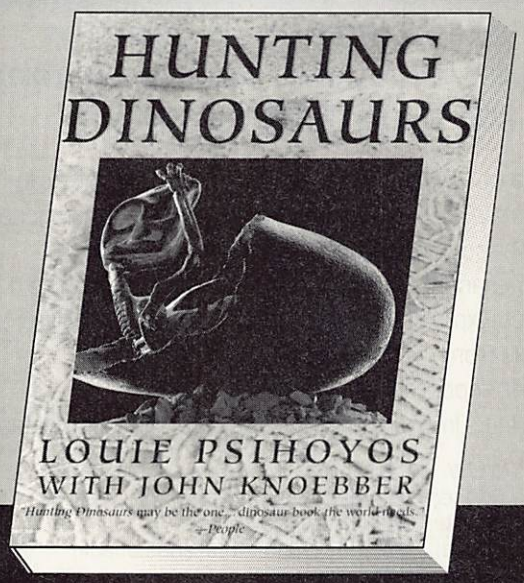

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E.M. Rubin, University of California

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Jennifer Lippincott-Schwartz, National Institute of Child Health and Human Development

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H. William Harris, Children's Hospital, Boston
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John D. Mountz, University of Alabama at Birmingham

Translational Research in Dermatology*
Juoni Uitto, Thomas Jefferson Medical College

Delivering Information to Clinicians in the Era of National Networking***
Edward H. Shortliffe, Stanford University School of Medicine
David Lipman, National Library of Medicine

Developmental Biology: Organogenesis***
Jeffrey A. Whitsett, Children's Hospital Medical Center, Cincinnati
Jeffrey I. Gordon, Washington University School of Medicine

The New Biology of Obesity
Jeffrey S. Flier, Harvard Medical School

Emerging Infections***
Ruth Berkelman, National Center for Infectious Diseases
Monica M. Farley, Emory University School of Medicine

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Eric G. Neilson, University of Pennsylvania
Robert J. Mayer, Harvard Medical School

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Original abstracts will be presented either in oral session, chaired by teams of senior investigators, or in poster sessions.

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Christine Seidman, Harvard Medical School

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Dorothea Zucker-Franklin, New York University

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George Bosi, Memorial Sloan Kettering Cancer Center

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William J. Koopman, University of Alabama at Birmingham

Gerontology/Aging
Mark A. Supiano, University of Michigan School of Medicine

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Leslie DeGroot, University of Chicago Medical Center

Renal/Hypertension
Barry M. Brenner, Harvard Medical School

Inflammation
William M. Nauseef, University of Iowa

Regulation of Gene Expression
Herbert H. Samuels, New York University

Physiology
J. Chris Gillin, University of California, San Diego

Cytokines/Growth Factors
Derek LeRoith, National Institute of Diabetes and Digestive and Kidney Diseases

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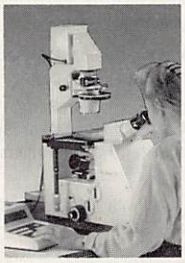

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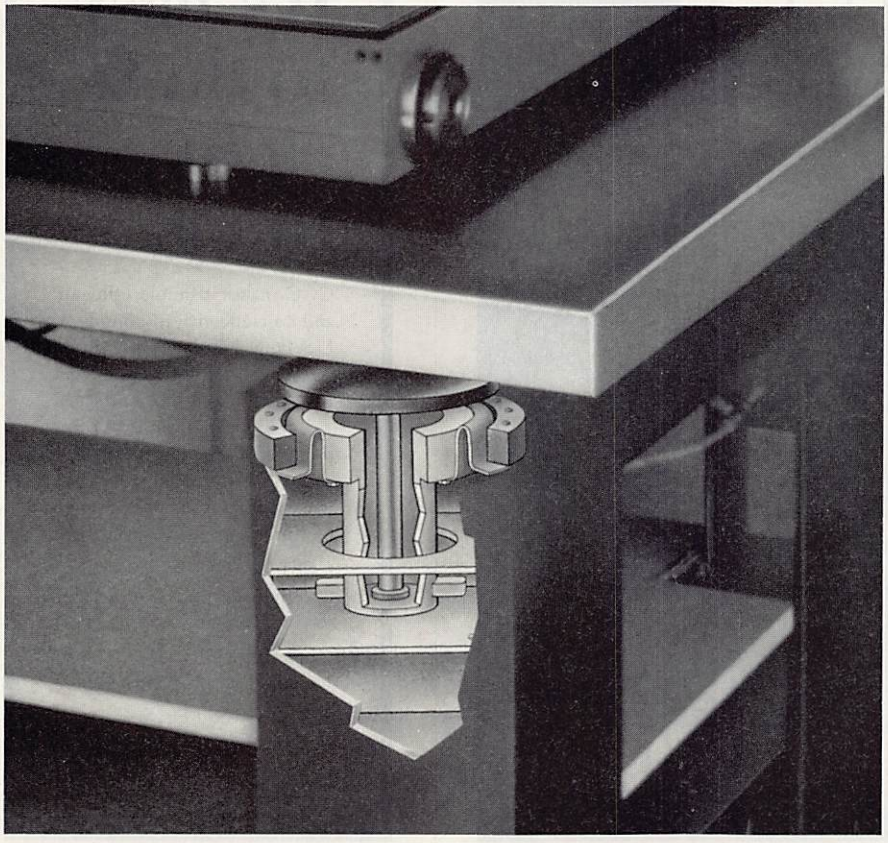

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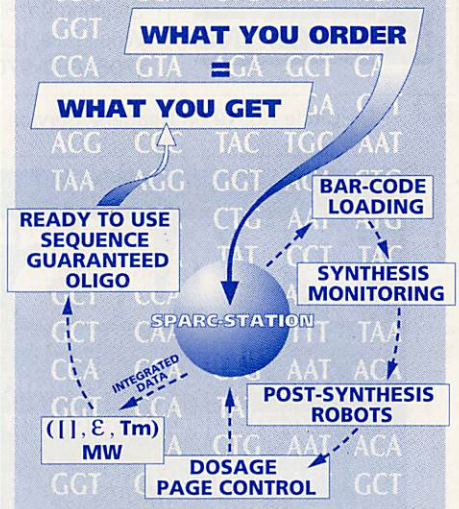

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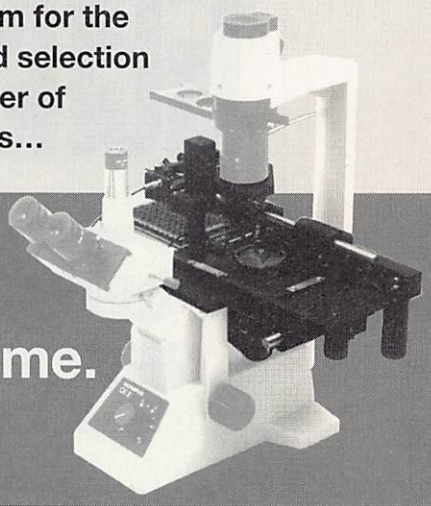

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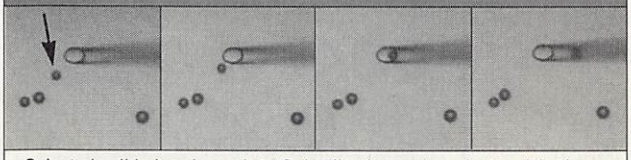

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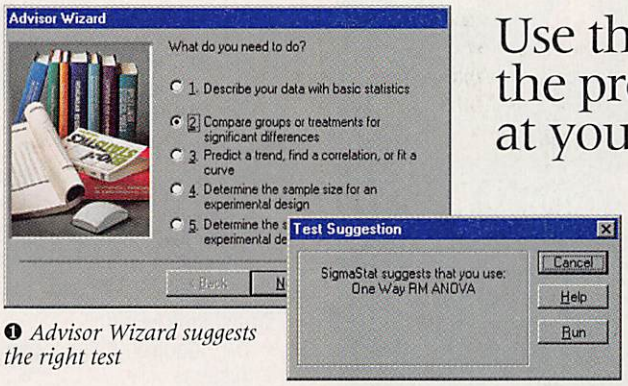
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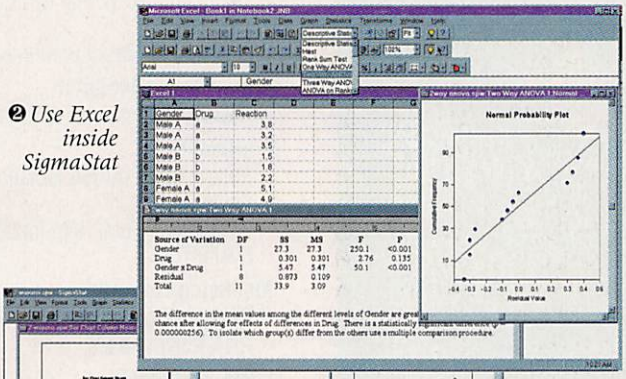

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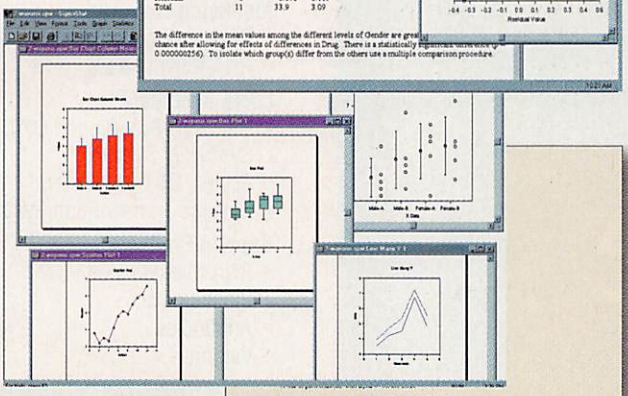

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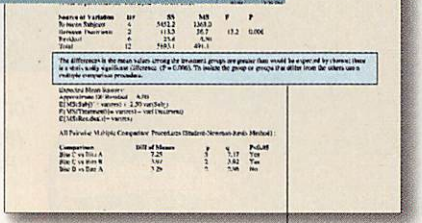

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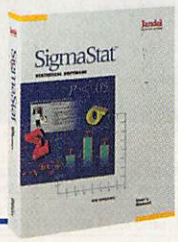

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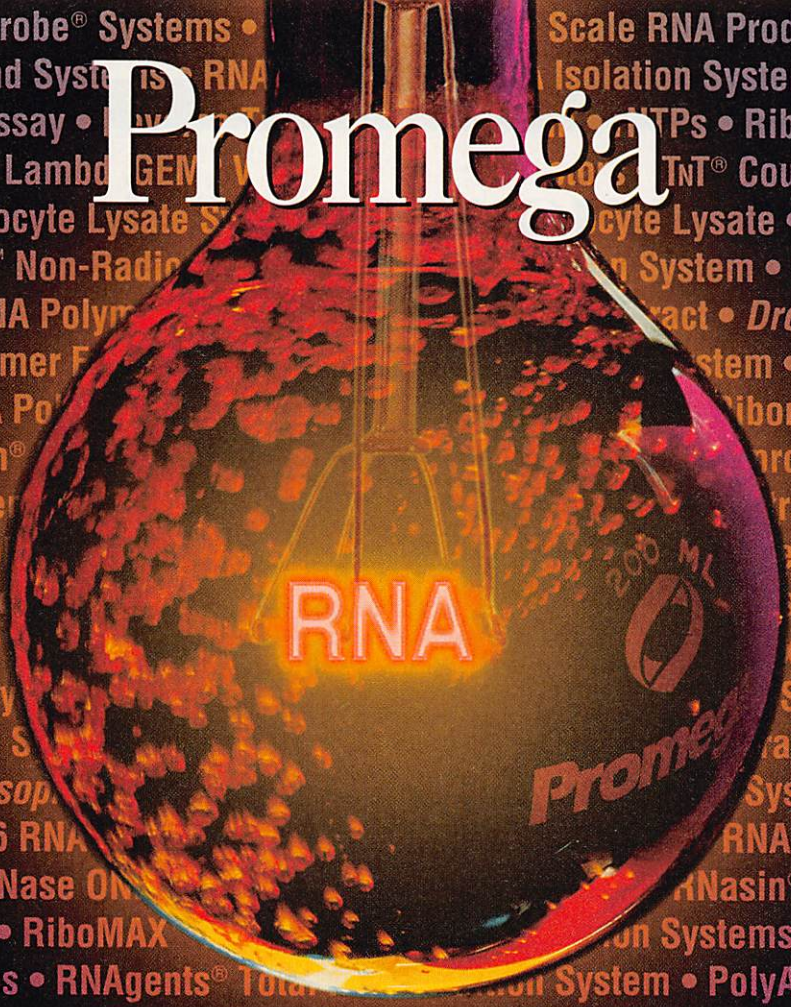

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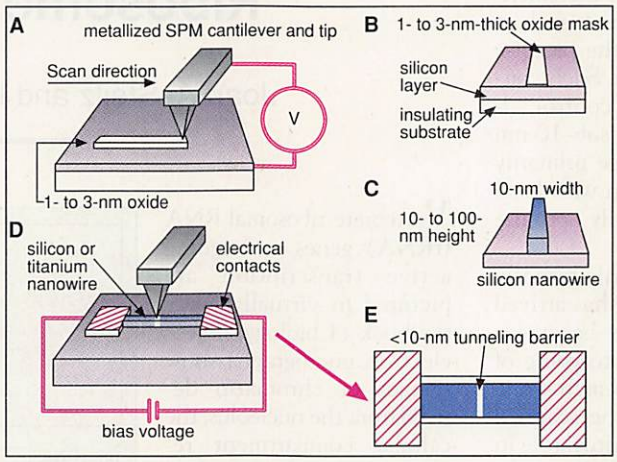
Device Fabrication by Scanned Probe Oxidation

J. A. Dagata

Nanoscience and nanotechnology have generated extraordinary interest in the past few years. This attention is largely a consequence of the invention of scanned probe microscopy (SPM) by Binnig and Rohrer (*1*). The development of SPM resonated with elements of Drexler's broad vision of nanotechnology, which itself was attracting considerable attention (*2*). The discovery that it is possible to use SPM not only for observing atomic-scale structure but also for manipulating material with atomic precision is a key element bridging SPM research activity and nanotechnology (*3*). After these initial scientific reports, significant efforts have been devoted to engineering functional nanometer-scale materials and devices by the direct use of SPM (*4*).

Progress in the case of one of these methods, the scanned probe direct oxidation technique, is particularly significant because of the remarkable level of cross-fertilization and incremental progress that has been achieved over the last few years by several laboratories worldwide. Snow and Campbell, on page 1639 of this issue, describe an important milestone in SPM fabrication of functional electronic device structures using this technique (*5*). Their work illustrates a defining feature of nanoscale engineering, that is, the integration of fabrication and measurement into a single process step is essential for attaining the desired performance. The collective experience and understanding needed to create a foundation for nanoscale engineering now exists. The profound simplicity and versatility of the direct oxidation technique has played a major role in making this possible.

Direct oxidation by SPM began at the U.S. National Institute of Standards and Technology (NIST) in early 1989 (*6*, *7*). The initial thrust of this work involved patterning single-crystal silicon and gallium arsenide surfaces. The basic idea is straightforward: Using a conducting SPM tip held either in contact with or within a nanometer of an electrically biased, stable, homogeneous substrate, one can induce a highly localized enhanced oxidation of the substrate (see figure). An oxide layer a few monolayers to a few nanometers in thickness rapidly forms in the substrate at the tip-sample junction. Typical linewidths are 10 to 20 nm, and writing speeds of up to 1 mm s^{-1} are possible, depending on the materials system, voltage bias between the tip and substrate, and available oxygen. Voltages used are 3 to 10 V, and both molecular oxygen and oxygen from adsorbed water layers, which exist under ambient conditions at the tip-sample junction, provide the reactant source. The presence of these impurities normally causes significant complications in other lithographic processes.



Thin oxide line. Scanning an electrically biased metallized SPM tip over the surface (**A**) can form a 1- to 3-nm-thick oxide line on many substrates. The pattern can then be used as an etch mask to create freestanding silicon nanowires (**B**,**C**). Further processing of the nanowire can also be carried out with the SPM tip (**D**) to create other confined structures.

The direct oxidation technique possesses remarkable features that are responsible for its success (*7*–*11*). (i) The method itself is simple to implement, relying on a process that is fairly well understood, because oxidation is a most ubiquitous chemical reaction. A number of metallic (titanium and chromium), semiconducting (crystalline, amorphous, and porous silicon; gallium arsenide), and insulating materials (SiO_2, Si_3N_4, and siloxanes) were patterned by this technique. (ii) The oxide is extremely stable relative to a typical native oxide. The conditions that exist at the junction ensure a strong driving force toward thermodynamically favorable bonding. (iii) The technique leads to sufficiently robust masks or final structures. The thermal and chemical stability required for subsequent processing by wet or dry chemical means is remarkable and, as a class, is probably unique to oxidation. The subsequent etching reactions required for forming nanostructures from the latent pattern for most materials means that a wide variety of materials are amenable to direct oxide patterning. (iv) The ability to operate at low voltage avoids proximity effects common to high-resolution electron-beam exposure. (v) Because of the method's simplicity and versatility, a diverse research base can implement these techniques, with some researchers taking an interest in the fabrication process itself and others using the process principally for device fabrication.

Steady progress has resulted in what has now become the fairly routine fabrication of functional devices. Technical improvements began with Snow and Campbell's use of metallized atomic force microscopy (AFM) tips in place of scanning tunneling microscopy (STM), which greatly improved reliability and increased writing speed, and with their demonstration that selective etching methods could be combined with epitaxial single-crystal silicon layers to form freestanding silicon structures up to 100 nm high from a latent pattern just 1 to 2 nm thick (*12*). They also demonstrated that silicon nanostructures are excellent masks for the etching of underlying heterogeneous layers, such as gallium arsenide and silicon dioxide (*13*). The next significant contribution occurred at Philips Laboratories in Europe: Researchers there extended this strategy of two-step etching by combining its use with amorphous silicon layers, which can be deposited onto a wide variety of metal and insulating films (*10*). This advance allowed the technique to be integrated into CMOS (complementary metal-oxide semiconductor) device processing, as first demonstrated by Quate's group at Stanford (*14*).

Meanwhile, Sugimura (now at Nikon in Japan) began investigating the oxidation of thin, titanium films (*8*). An advantage of this system is that when the film thickness was carefully controlled to 5 nm or less on an insulating substrate, SPM direct oxidation rendered exposed regions of the metallic film insulating. This advance made critically dimensioned device components possible in a single step through the formation of insulating constrictions in conducting titanium wires. Matsumoto and co-workers at the Electrotechnical Laboratory in Japan

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reported device characteristics for a single-electron tunneling transistor (SET) junction formed in this way (*15*). Although the SET performance of these devices appears entirely convincing, the correlation of device size and quantum confinement has not yet been confirmed on devices based on titanium wires, which presumably reflects a contribution of grain size on performance. Problems such as these, where reliable control of electrical and dimensional properties at less than 10 nm becomes critical, demand that fabrication be coupled with in situ diagnostics. By monitoring a resistance increase in the wire during fabrication, Snow and Campbell have shown that routine and reliable control can be attained in this size regime (*5*). A distinguishing feature of SPM-based nanoscale technology, that which sets it apart from the incremental refinements of existing methods such as electron-beam lithography, is the promise of exceptional materials control. Such control, rather than linewidth control, is needed for investigations at the sub–10-nm level. Further progress will hinge primarily on the integration of fabrication with local diagnostic probes in order to fully optimize the process (*16*).

Direct oxidation by SPM, only recently at the frontiers of nanoscience, has arrived at the point where it has now become a powerful technique for the prototyping of novel device structures. This research is one aspect of a generalization of the scanned probe concept leading to its eventual role in advanced manufacturing. Multiple-tip arrays, in which large numbers of individual SPM instruments perform parallel fabrication and inspection over a large sample area, are now a reality. Quate's group at Stanford has demonstrated parallel lithography by coupling the direct oxidation process with a five-tip SPM array (*17*). As these developments continue to be explored in the research laboratory, industrial acceptance of scanned probe techniques is growing rapidly. Various types of critical measurements needed in production and process development for the semiconductor, magnetic recording, and polymers and coatings sectors have been identified (*18*). These applications have the potential to drive the creation of an integrated SPM-based technology employing both fabrication and measurement functions. The prospects for this will be determined largely by the extent to which nanoscience research and technological innovation become successful at anticipating each other's development paths.

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Small RNA Chaperones for Ribosome Biogenesis

Joan A. Steitz and Kazimierz T. Tycowski

Vertebrate ribosomal RNA (rRNA) genes undergoing active transcription are pictured in virtually every textbook of biology. These electron micrographs show spreads of chromatin derived from the nucleolus, the cellular compartment responsible for ribosome biogenesis. The nascent rRNA molecules that emanate from the DNA pinpoint the sites where synthesis begins and ends within the tandem repeats of rDNA (*1*). But these elegant visualizations of genes in action also convey an important message concerning the fate of the attached precursor rRNA (pre-rRNA) transcripts (Fig. 1). Because their lengths uniformly increase along each transcription unit [~13,000 nucleotides (nt) in mammals], significant RNA processing must not occur until synthesis is complete. Approximately half of pre-rRNA sequences are then discarded during the maturation of 18S, 5.8S, and 28S rRNAs (*2*). Although ciliates splice their pre-rRNAs, the processing pathway in vertebrates consists simply of an ordered series of endo- and exonucleolytic cleavages that carve away the spacer regions and trim the ends of the rRNAs. What nucleolar components orchestrate these cuts, and why do cells complete synthesis of an enormous RNA before embarking on the process?

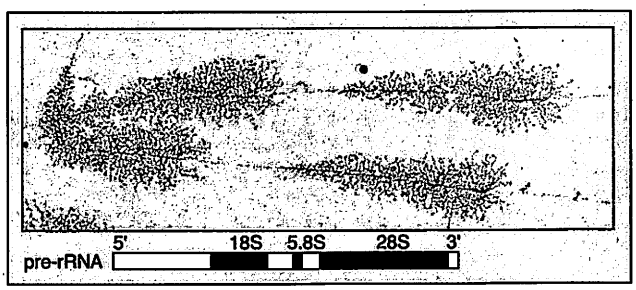

Fig. 1. Electron micrograph of actively transcribed rRNA genes from the *Triturus viridescens* oocyte. Each transcription unit shows growing pre-rRNA chains decorated at their 5′ ends with the so-called terminal knobs. Lower panel shows a schematic representation of vertebrate pre-rRNA. [Micrograph courtesy of O. L. Miller.]

Small nuclear ribonucleoproteins (snRNPs) have recently been identified as major players in eukaryotic rRNA processing (*3*). In addition to the well-studied snRNPs that assemble into spliceosomes to excise introns from pre–messenger RNAs (pre-mRNAs), cells contain distinct snRNPs localized in the nucleolus. The small RNAs in vertebrate nucleolar snRNPs range in length from 70 to more than 200 nucleotides. Many of these snRNPs contain a protein called fibrillarin, an autoantigen that is an abundant component of the fibrillar regions of the nucleolus. Current estimates of the number of nucleolar snRNPs in both vertebrates and yeast range from 50 to 100; approximately 20 vertebrate RNA sequences are now known.

So far, only three of the many vertebrate nucleolar snRNPs have been assigned functions in rRNA processing (*3*). The most

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abundant of these (~10^6 molecules per cell), U3, is essential for an early cut close to the 5′ end of the transcript, curiously distant from any of the mature rRNA sequences. It then remains attached to the downstream product, generating the distinctive 5′-terminal knobs that characterize rRNA (but not mRNA) transcripts in chromatin spreads (*4*). Binding of the U3 snRNP might therefore be considered to commit the rRNA transcript to the processing pathway; in yeast, this interaction is essential for the appearance of 18S rRNA (*5*, *6*). The second most abundant nucleolar snRNP, U8, is required for cleavage upstream of 5.8S rRNA as well as at the 3′ end of 28S rRNA, ~5000 nt away (*7*). The less abundant U22 snRNP is essential for processing at both the 5′ and 3′ ends of the 18S rRNA, separated by ~2000 nt (*8*). Functions for two additional vertebrate snRNPs can be extrapolated from studies of their counterparts in yeast (*3*). U14 is needed for maturation of 18S rRNA (*9*), whereas RNaseMRP (a relative of the RNaseP ribozyme) executes cleavage upstream of 5.8S sequences (*10*). Unfortunately, establishing which vertebrate small nucleolar RNAs are homologous to the many other known yeast small RNAs has not been possible.

The mechanistic details of how the nucleolar snRNPs act on the pre-rRNA remain elusive. But the surprising realization that a single snRNP can coordinate cuts at far distant sites allows pleasing parallels to be drawn with bacterial rRNA processing. There, complementary sequences flanking the 5′ and 3′ ends of the 16S and 23S rRNAs come together, generating extensive stems in the pre-rRNA that are cleaved by a protein enzyme, ribonuclease III (*11*). In eukaryotes, comparable stems are not apparent, but the snRNP-dependent coupling of processing at remote sites flanking the large or small subunit rRNA (*7*, *8*, *10*) must reflect a similar pre-rRNA architecture.

The task of folding the huge eukaryotic pre-rRNA transcript may well be accomplished by a myriad of small nucleolar RNAs discovered in the last several years (*3*, *12*). The vast majority of these RNAs in vertebrates are generated by an unprecedented biosynthetic route. Their genes are embedded within the introns of protein-coding genes, most of which are involved in ribosome or nucleolar functions (*3*). Apparently, the host intron is excised from the pre-mRNA transcript and debranched; then, the small RNA is released by exonuclease action (*13*). Most bind the common nucleolar snRNP protein fibrillarin, directed by conserved sequences (called boxes C and D) adjacent to a stem formed by the termini of the intron-encoded small RNA.

The truly striking feature of the intron-encoded small nucleolar RNAs, however, is their complementarity to mature rRNA sequences. Each little RNA has the potential to form a nearly perfect duplex of 11 to 21 base pairs with either 18S or 28S rRNA (*12*, *14*). Usually, the complementarity lies within one of the universal core secondary structures that have been deduced from phylogenetic comparisons of the large rRNA sequences (Fig. 2). Association of snRNP would therefore disrupt rRNA stems or other conserved interactions, conferring an alternative geometry on the pre-rRNA undergoing processing. In a few cases, psoralen cross-linking (*15*) or genetic suppression studies (*16*) have provided direct evidence for the predicted snRNP-rRNA base pairing. The cellular abundance of the new nucleolar snRNPs is similar to that estimated for pre-rRNA (~10^4), compared with 10^7 ribosomes in the mammalian cytoplasm (*3*). In localization studies, the small RNAs are exclusively nucleolar, suggesting that they recycle within this nuclear subcompartment. The proposed role of nucleolar snRNPs in remodeling the structure of the large pre-rRNA transcript therefore mimics the action of protein chaperones in directing the folding of other polypeptide chains (*3*, *14*).

The chaperone analogy raises questions nearly as numerous as the new nucleolar snRNPs themselves (*3*, *14*). How many are there? How many are essential, as opposed to having overlapping or facilitating functions? Is adenosine triphosphate hydrolysis required for their release (as with protein chaperones)? Or are they displaced simply by ribosomal proteins assembling on the nascent rRNAs? Do they direct the relatively rare nucleotide modifications (pseudouridine and base or sugar methylations) that are likewise clustered within the universal core regions of the rRNAs (*14*)? Do they serve not only to produce a specific geometry for the pre-rRNA transcript, but also to preclude deleterious intermolecular interactions between the GC-rich, closely spaced neighboring transcripts? If nucleolar snRNPs explain repeated failures to reconstitute eukaryotic ribosomal subunits from their constituent rRNAs and proteins, how can bacterial ribosomes assemble without the assistance of small RNA chaperones? Whether or not nucleolar snRNPs are eventually assigned catalytic activities, their existence adds the nucleolus to the growing roster of cellular RNA machines.

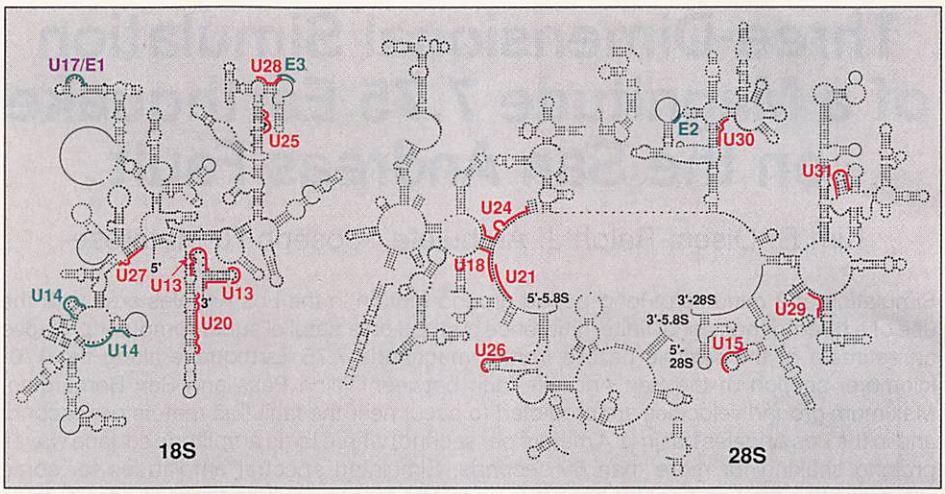

Fig. 2. Base-pairing interactions of small nucleolar RNAs with 18*S* and 28*S* rRNAs. Red, predicted base pairings; purple, experimentally supported base pairings; darker balls, rRNA nucleotides that are more than 95% identical among eukaryotic species; lighter balls, nucleotide positions that exist 95% of the time; continuous lines, regions highly variable in size and content.

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Three-Dimensional Simulation of a Magnitude 7.75 Earthquake on the San Andreas Fault

Kim B. Olsen, Ralph J. Archuleta,* Joseph R. Matarese

Simulation of 2 minutes of long-period ground motion in the Los Angeles area with the use of a three-dimensional finite-difference method on a parallel supercomputer provides an estimate of the seismic hazard from a magnitude 7.75 earthquake along the 170-kilometer section of the San Andreas fault between Tejon Pass and San Bernardino. Maximum ground velocities are predicted to occur near the fault (2.5 meters per second) and in the Los Angeles basin (1.4 meters per second) where large amplitude surface waves prolong shaking for more than 60 seconds. Simulated spectral amplitudes for some regions within the Los Angeles basin are up to 10 times larger than those at sites outside the basin at similar distances from the San Andreas fault.

The damage in Mexico City from the Michoacan earthquake (19 September 1985) and in the Marina district of San Francisco from the Loma Prieta earthquake (19 October 1989) has clearly illustrated the risks for population centers located in basins at a significant distance from the causative fault. The sediments filling the basins, which have low elastic moduli, amplify the seismic waves relative to the surrounding bedrock. In addition, the edges of the basins can generate large-amplitude waves that can prolong the shaking in the basins. Moderate-sized events, such as the magnitude (**M**) 6.7 Northridge earthquake (17 January 1994), the **M** 6.0 Whittier Narrows earthquake (1 October 1987), and the **M** 6.6 San Fernando earthquake (9 February 1971), have emphasized the seismic hazard from faults on the Los Angeles (LA) fault system (*1*–*5*). Nonetheless, the San Andreas fault (SAF) remains potentially the most hazardous for the LA area because it has produced large earthquakes in historical time (*2*, *6*, *7*).

To determine the amount of shaking that could occur in the LA area, we simulated a **M** 7.75 earthquake on the section of the SAF closest to LA. We considered a propagating rupture for 170 km from Tejon Pass to San Bernardino (Fig. 1A). This part of the SAF, which consists of two segments, the Mojave and part of the San Bernardino Mountains segment (*2*), is believed to have produced the **M** 7.5 earthquake on 12 December 1812 (*6*, *8*). The average recurrence time between large earthquakes with surface rupture on the Mojave segment is 150^{+123}_{-71} years and on the San Bernardino segment it is 146^{+91}_{-60} years (*2*). Mean conditional probabilities of 26 ± 11% (**M** 7.53) and 28 ± 13% (**M** 7.30) have been determined for earthquakes on the Mojave and San Bernardino segments, respectively, to occur before the year 2024 (*2*). Because both segments have similar recurrence intervals and conditional probabilities, and may have ruptured together in earlier events (*6*), it is reasonable to consider a scenario where this section of the SAF ruptures again in a single earthquake.

To estimate the ground shaking from such an earthquake (*9*) we incorporated the three-dimensional (3D) structure of the medium through which the seismic waves will travel from the SAF to LA. The geology of this region is varied: Igneous and metamorphic basement rocks crop out extensively in the Mojave Desert and San Gabriel Mountains (*10*) whereas sedimentary rocks fill the deep basins in LA and the San Fernando Valley (*11*). The seismic energy will thus propagate for at least 35 km through igneous and metamorphic rocks before impinging on the sedimentary basins of greater LA (Fig. 1B). Were it not for the basins beneath the San Fernando Valley and LA (including the San Gabriel Valley) (Fig. 1B), the model would consist of horizontal layers where the material parameters varied only with depth (*12*). However, with their irregular geometry and different material properties, these sedimentary basins affect the ground motion by amplifying seismic waves, prolonging their duration, and generating waves at the basin edges (*3*, *13*, *14*).

To approximate a **M** 7.75 earthquake on the SAF, we simulated a rupture on a fault plane 16.5 km deep and 170 km long that extends from Quail Lake (10 km southeast of Gorman) to Mill Creek (11 km northeast of Redlands). The fault plane had a constant strike (118°) that followed the strike of the SAF with a slight deviation (maximum of 6 km) at the northwestern end of the Mojave segment (Fig. 1A). The rupture initiated at a point 2 km from the northwestern end of the fault segment, at 10 km depth, and dextrally offset the two sides of the fault by 4.82 m everywhere (*15*) (Table 1). The earthquake rupture lasted 68 s before terminating at Mill Creek. The shear (S) waves that continuously radiated from the rupture impinged on the basins about 20 s after the earthquake started. Some areas of the LA basin continued to vibrate for more than 60 s because of the continual stimulation from waves arriving from different parts of the fault, waves generated at the basin edges, and resonances in the basin.

Numerical model for the earthquake rupture and 3D wave propagation. We subdivided a large volume (230 km by 140.4 km by 46 km) of southern California into 23,209,875 cubes 0.4 km on a side with a gridpoint at each vertex. Each gridpoint was assigned a compressional wave velocity, an S wave velocity, and a density (*16*). The 3D structure did not include the basins beneath the Upper Santa Ana Valley, the San Bernardino Valley, or the Ventura area (*14*, *17*) because there was no discretized 3D model for their elastic and material properties. Therefore the amplitude and duration of the simulated ground motion is probably underestimated in these areas (*14*, *18*).

We kinematically simulated the earthquake as a constant slip that radially propagated outward with a velocity 85% of the local S wave velocity. The slip rate function is Gaussian with a dominant period of 4.5 s. This function was later deconvolved from the synthetic records to obtain a slip rate with uniform spectral response to displacement for frequencies up to 0.4 Hz that is constant everywhere on the fault (*3*). This formulation allows the seismic moment and synthetics to be scaled by a single value of slip. The effective rise time is about 3 s everywhere on the fault (*19*).

The source was implemented in the finite-difference grid by adding $-M_{ij}(t)/V$ to $S_{ij}(t)$, where $M_{ij}(t)$ is the ij'th component of the moment tensor for the earthquake, V is

Table 1. Earthquake rupture parameters.

Southeast fault limit	34.089°N, 117.064°W
Northwest fault limit	34.808°N, 118.688°W
Hypocenter	34.800°N, 118.669°W
Hypocenter depth (km)	10
Slip (m)	4.82
Moment magnitude (**M**)	7.75
Width, length (km)	16, 170
Depth to top of fault (km)	0.5
Dip, strike, rake (°)	90, 118, 180

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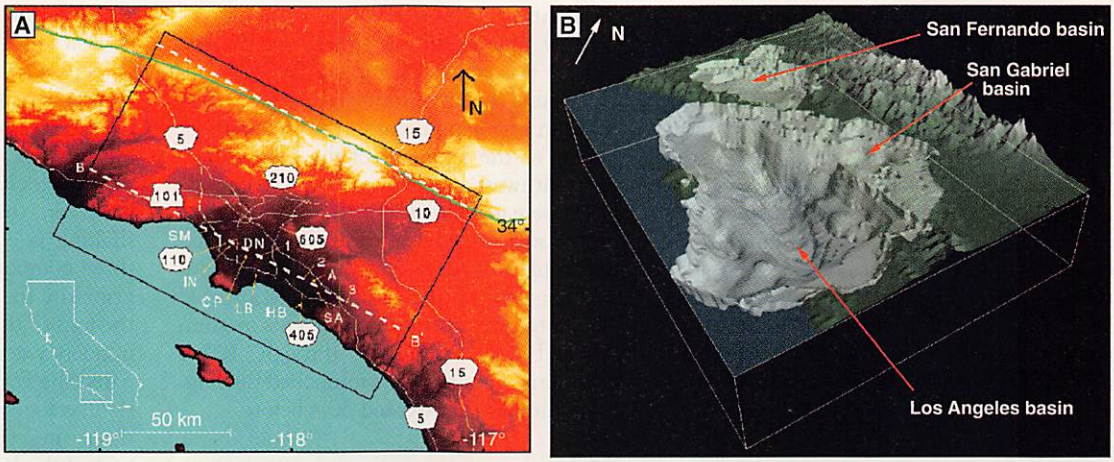

Fig. 1. (**A**) Topographic map of southern California. The rectangle shows the areal extent of the simulation. The SAF is shown by the green line; our approximation to the SAF is shown by the dashed white line. The thick solid white line is the coastline of southern California. The thin white lines depict major freeways. The dashed white line B-B′ is a profile used for displaying seismograms. 1, 2, and 3 denote sites in the basin, on the edge of the basin, and outside the basin, respectively. A, Anaheim; CP, Compton; DN, Downey; HB, Huntington Beach; IN, Inglewood; SA, Santa Ana; and SM, Santa Monica. (**B**) Three-dimensional perspective of the isosurface for an S-wave velocity of 2.6 km/s illustrating the geometry of the basins. Vertical exaggeration is 3.2. Modeling parameters are listed in Table 1.

the cell volume, and $S_{ij}(t)$ is the ij'th component of the stress tensor on the fault at time t (Table 2).

We use a staggered-grid finite-difference scheme to solve the 3D elastic equations of motion (*20*); the accuracy is fourth order in space and second order in time. The numerical implementation of the 3D scheme is described in (*21*). To eliminate artificial reflections from the boundaries of the grid, we implemented absorbing boundary conditions coupled with a buffer zone of strong attenuation (*22*). In the following, we removed these zones and present the simulation results within the area (174 km by 114.8 km) (Fig. 1A) that encompasses the population centers of the LA area.

Finite-difference modeling of elastic waves in large-scale 3D models, such as the one used in the SAF simulation, consumes vast quantities of computational resources. The earth model and the calculated stress and velocity fields require gigabytes of physical memory and gigaflops of CPU for tens of hours to simulate the total duration of ground motion within a reasonable period of time. Such computational requirements are beyond the resources of workstations and most supercomputers with shared-memory configurations. To carry out our simulation we used grid-decomposition techniques on the massively parallel processors of the nCUBE 2 at the Earth Resources Laboratory of the Massachusetts Institute of Technology.

Table 2. Three-dimensional modeling parameters.

Spatial discretization (km)	0.4
Temporal discretization (s)	0.025
Minimum P-wave velocity (km/s)	2.41
Minimum S-wave velocity (km/s)	1.00
Minimum density (kg/m^3)	2070
Gridpoints along 118°	576*
Gridpoints along 28°	352*
Gridpoints along vertical	116
Timesteps	4800
Simulation time (s)	120

*Figures show 436 gridpoints along the 118° azimuth and 288 gridpoints along the 28° azimuth.

Figure 2 illustrates how we subdivide the 3D volume of the model over eight parallel processors (Fig. 2). Each processor is responsible for performing stress and velocity calculations for its portion of the grid, as well as dealing with boundary conditions at the external edges of each volume. At the internal edges, where neighboring portions of the earth volume are contained on separate nodes, the processors must exchange stress and velocity information to propagate the waves correctly. On the nCUBE 2, this communication time represents a negligible part of the total run time. In other words, the same simulation can be performed twice as fast on twice as many processors as long as the internal edges represent a small part of the model held by each processor.

For the SAF simulation we used all 512 processors available on the nCUBE 2. With grid dimensions of 576 by 352 by 116 points, the most memory-efficient decomposition involved assigning subgrids of 36 by 44 by 29 points to the individual processors. In keeping with the fourth order finite-difference scheme, a two-point-thick padding layer was added to the outside of each subgrid to bring the total subgrid dimensions to 40 by 48 by 33 points. To store 12 arrays (three elastic coefficients, three components of velocity, and six components of stress) of this size required just over 2.9 megabytes of the 4 megabytes of physical memory on each processor. The remaining memory was consumed by the program logic and additional variables as well as the operating system kernel. Using a grid decomposition code based upon the portable Message Passing Interface standard, the finite-difference simulation took 17 s per timestep or nearly 23 hours to complete the 120 s of simulated ground motion. More than a gigabyte of disk space was used to store the time history of the ground motion at the surface.

Simulated ground motion and seismic hazard in LA. To illustrate the development of the ground motion as the rupture propagated along the SAF we show snapshots of long-period ground velocity (Fig. 3) over the 19,975 km^2 area (Fig. 1A). By 20 s, the S waves had entered San Fernando Val-

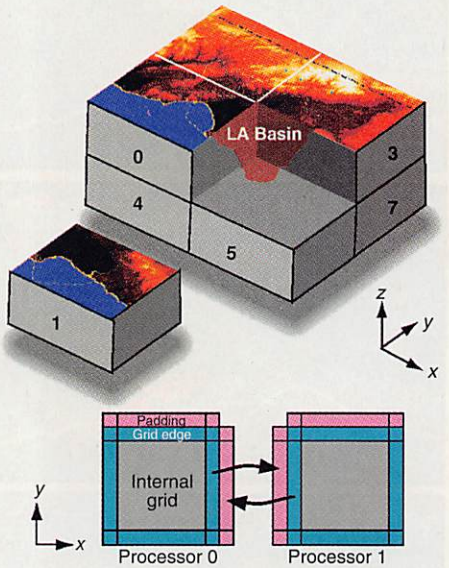

Fig. 2. Grid decomposition of the SAF rupture model on the nCUBE 2. (**Top**) The model is decomposed into a number of subgrids, labeled 0 through 7. The actual model was divided into 512 subgrids, corresponding to the number of processors used in the simulation. (**Bottom**) Illustration of the communication between neighboring subgrids. The fourth-order finite-difference scheme requires that a two-point-thick padding layer be added to the outside of each subgrid in order to propagate the waves correctly. This padding layer always contains the most recently updated wavefield parameters exchanged from the edge of the neighboring subgrid (arrows).

ley. By 30 s, when the rupture had propagated about halfway along the SAF, the S waves had entered the main LA basin. The area near the northern intersection of Interstates 5 and 405 showed intense ground motion. The high intensity was produced because resonance in the deep part of the San Fernando Valley basin radiated secondary waves as seen by the faint circular wavefronts centered on this area (R, Fig. 3 at 40 s). At 40 s, the ground motion in the LA basin had intensified: the San Gabriel Valley, just southeast of the intersection of Interstates 10 and 605, and the area just south of downtown LA show large amplitude ground motions. At 50 s the entire LA basin was excited by large amplitude surface waves. The rupture was almost complete by 60 s, at which time the wavefronts in the basin slowly began changing direction from southeast parallel to the long axis of the LA basin to the southwest. At 70 s, large amplitude waves were generated by reflections from the steep northeast sides of the basin; the peaks and troughs of the waves were aligned with the long axis of the basin and propagated west into the offshore area. This pattern persisted but diminished through 100 s where only faint shadows mimicked the larger amplitudes that existed 30 s before.

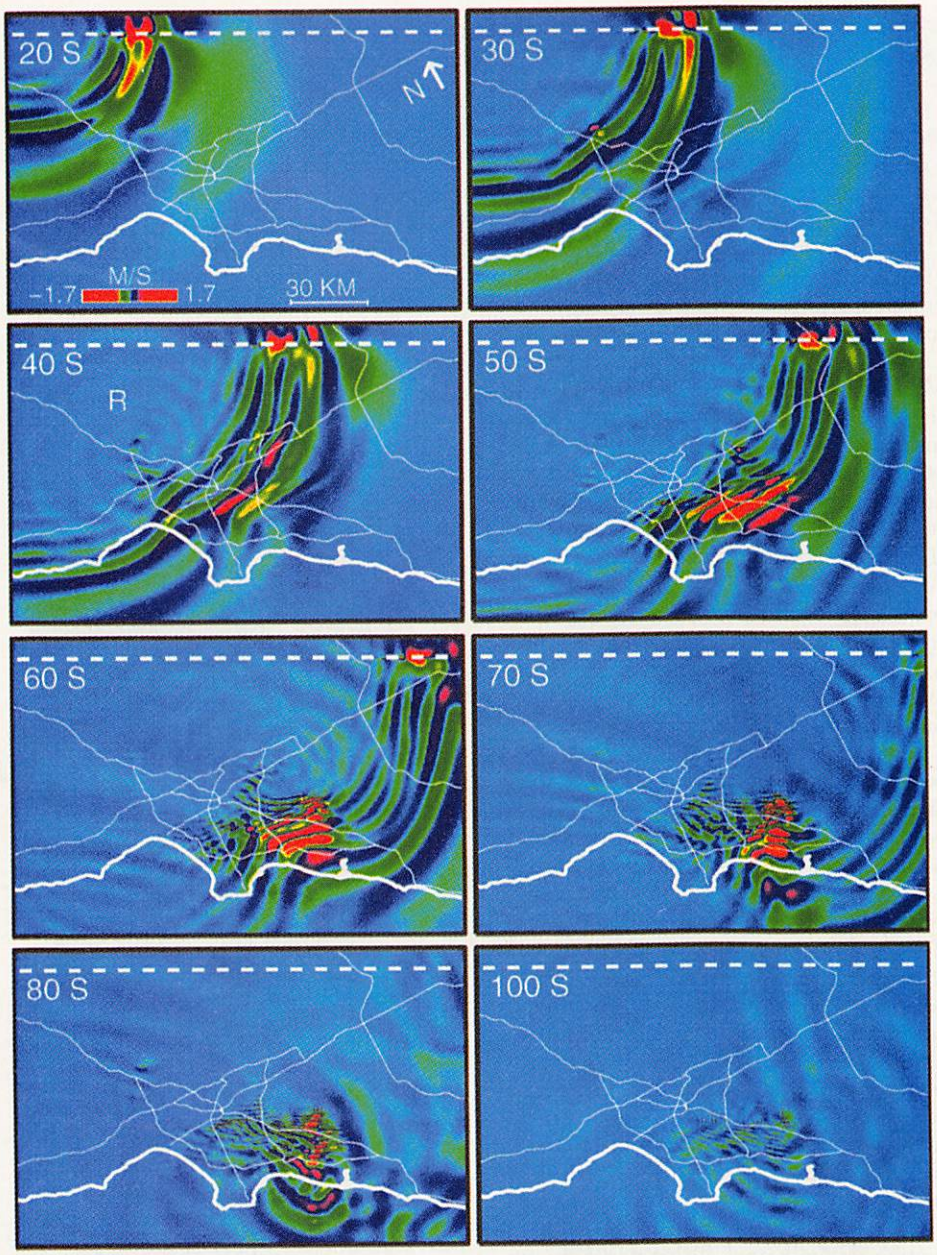

Fig. 3. Snapshots of simulated wave propagation in the LA area for the hypothetical SAF earthquake; the snapshots depict the horizontal (118°) particle velocities that represent shaking parallel to the SAF (*23*) from 20 s to 100 s after the origin time of the rupture. Red depicts large amplitudes of both positive and negative polarity. R depicts an area of local resonance above the deepest part of the San Fernando Valley basin. The particle motion is scaled by a constant for all snapshots. Lines labeled as in Fig. 1A.

To encapsulate many of these effects we show a suite of seismograms and the S wave velocity structure (Fig. 4, A and B) from the Ventura basin to the mountains east of San Clemente (B-B′, Fig. 1A). By visually correlating the suite of seismograms with the S wave velocity structure, the effect of the basin on the amplitude and duration of shaking is evident. The basin affected all three components of motion (Fig. 4C) but primarily the component parallel (118°) to the axis of the trough (*23*). To examine the frequency content of the shaking, we compared the Fourier spectral amplitudes of particle velocity for the 118° component for three sites (Fig. 4D): (i) over the deepest part of the basin, (ii) above the edge of the basin, and (iii) outside the basin (Fig. 1A). The maximum spectral amplitude for the edge site was a factor of two larger than for the site over the deepest part of the basin. The edge site had its spectral maximum at a higher frequency (0.25 Hz) compared to the site above the central basin where the first broad peak occurred around 0.16 Hz—approximately the fundamental resonant frequency of the LA basin (*24*). The spectral amplitude for a site at the edge of the basin was 10 times the spectral amplitude for a site outside the basin even though both are at the same distance from the SAF (*25*).

Contours of the peak particle velocity over the area reveal effects due to rupture and structure (Fig. 5). The 118° component was largest near the SAF and in the LA basin. The high amplitudes near the fault were expected; the amplitudes (>0.5 m/s) in the LA basin ~60 km from the fault were not. The 0.25 m/s contour basically outlines the San Gabriel Valley and LA basins. Moreover, there is a large area with amplitudes larger than 0.75 m/s corresponding to the deeper parts of the LA basin.

The large lobe with peak velocity greater than 0.75 m/s for the 28° component (Fig. 5) reflects the directivity of the rupture (*4*, *26*) and the radiation pattern from the source (*23*) that combine to produce the maximum slip velocity 2.5 m/s. The 28° component shows that in the simulation those areas in the forward direction of the rupture were as severely shaken as LA. Most of the area with peak velocity greater than 0.75 m/s was confined to the less populated San Gabriel Mountains. However, the southeastern part of the 0.75 m/s lobe cov-

Table 3. Comparison of maximum peak particle velocities (m/s) near the SAF and in the LA basin for three components of wave motion.

Location	118°	28°	Vertical
Near SAF	2.01	2.51	0.91
LA basin	1.37	0.70	0.54

ered the region in the populated Upper Santa Ana Valley (including cities such as Ontario, Pomona, and Upland) and the San Bernardino Valley (including cities such as San Bernardino and Redlands). Because the SAF passes just to the northwest of these areas, the severity of the shaking increases because of proximity to the fault (*25*) and rupture directivity. The amplitude of the waves attenuated with distance from the SAF except for the amplification resulting from the LA basin where amplitudes on the 28° component also exceeded 0.5 m/s.

The smallest peak particle velocities were for the vertical component as expected for strike-slip motion on a vertical fault. However, like the horizontal components, the LA basin amplifies the vertical motion relative to the surrounding area. The net result is that the total ground motion in the LA region in the simulation was much larger than would be predicted by a 1D model (*3*, *13*, *14*). We illustrate this point by mapping the total cumulative kinetic energy (*27*) that takes into account the amplitude and duration of shaking for all three components of motion (Fig. 6). A large swath within 30 km of the SAF experienced significant shaking. The cumulative kinetic energy decreased with distance from the fault except for the basins, especially the LA basin—where, for frequencies up to 0.4 Hz, the cumulative kinetic energy was equivalent to that near the fault. The expected attenuation with distance of the amplitude of the seismic waves (*25*) was counteracted by the basin geometry and velocity structure.

The simulated ground motion should be considered in the context of three factors: (i) Because of computational limitations, the maximum frequency was 0.4 Hz. Observed ground motions contain higher frequencies that will increase the amplitudes of the particle velocities especially near the SAF where anelastic material attenuation has less effect than for distances far from the fault (*25*). The component of motion perpendicular to the strike (28°), is 25% larger than the parallel component near the fault (Table 3). This 28° component is amplified by directivity of the rupture and almost always has the maximum particle velocity (*4*, *26*). However, the observed near-fault peak velocities were generally associated with pulses dominated by frequencies 1.0 Hz or less (*4*, *26*) so that the lower frequency of 0.4 Hz may not be a critical factor. The structures most affected by the computed ground motion would be those that have their lowest mode response with frequencies less than 0.4 Hz (periods ≥2.5 s); almost all one- and two-story structures have their peak response at much higher frequencies (*28*). (ii) The minimum S wave velocity is 1.0 km/s, again due to computational limitations. In engineering terms this means that all of the ground motion is for rock rather than soil (*29*). Inclusion of the lower velocity sediments would increase the ground motion amplitudes (*30*). The omission of higher frequencies and lower near-surface velocities in the simulation define our results as a lower bound for the expected ground motion for the SAF rupture scenario. (iii) Our kinematic rupture model is smooth in that the slip and slip rate are constant everywhere on the fault and rupture velocity is a fixed percentage of the S wave velocity. The slip and the slip rate have plausible values (*15*) but could certainly be allowed to vary spatially; such variation would affect signal duration more than amplitude (*9*). In real, large earthquakes rupture velocity varies significantly over long fault length. A variable rupture velocity would decrease the coherency of the waves leaving the fault and mitigate the effects of directivity. Whether this would increase or decrease the ground motion in LA is unknown.

Our simulation of a **M** 7.75 earthquake on the SAF predicted long-period peak par-

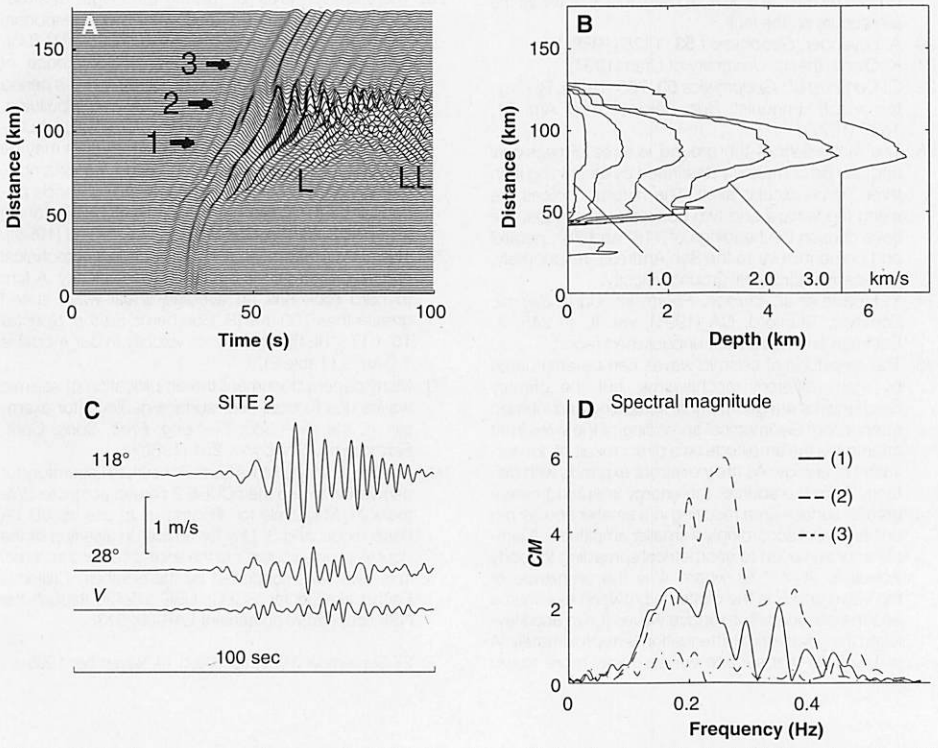

Fig. 4. Seismic response along profile B-B′ (see Fig. 1A) for the hypothetical SAF earthquake. (**A**) Simulated velocity seismograms for the 118° horizontal component: site 1 is within the basin, site 2 is along the basin edge, and site 3 is outside the basin. (**B**) Vertical cross section along B-B′; the contours on the map depict the S-wave velocity at a contour interval of 0.5 km/s. (**C**) Three component velocity seismograms at site 2. L depicts Love waves and LL depicts Love waves reflected at the basin edge. (**D**) Fourier spectral amplitude for the 118° horizontal component at sites 1, 2, and 3 (see Fig. 1A).

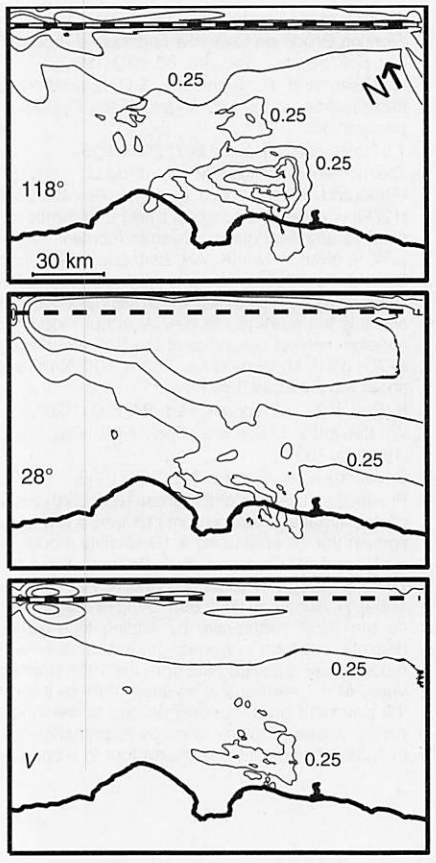

Fig. 5. Contour maps of simulated peak velocities along the 118°, 28°, and vertical components for the hypothetical SAF earthquake. Contour interval is 0.25 m/s with the 0.25 m/s contour labeled. Lines labeled as in Fig. 1A.

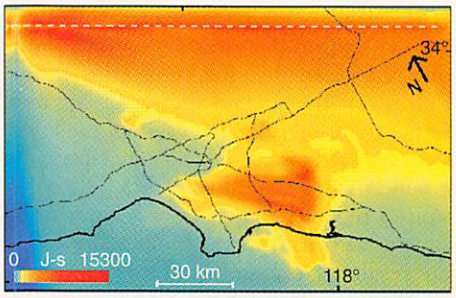

Fig. 6. Map of simulated total cumulative kinetic energies for the hypothetical SAF earthquake. Note that the color scale is logarithmic in units of Joules-seconds (J-s). Lines labeled as in Fig. 1A.

ticle velocities greater than 1.0 m/s at some areas in LA and greater than 0.75 m/s over a large central area even though the main trough of the basin is about 60 km from the fault (Fig. 5). These peak velocities are similar to those observed near the fault for some damaging earthquakes (*4*, *26*) and larger than particle velocities from simulated M 6.75 earthquakes on faults in LA (*3*). While these simulated long-period waves have large amplitudes, the threat they pose to existing structures is unknown.

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19. A propagating rupture is analogous to a double zipper. The first zipper, moving at the velocity of the rupture, unlocks the two sides of the fault. A second zipper arrives later to lock the two sides of the fault. In the time between the two zippers, the two sides of the fault move relative to each other. The total amount of relative movement before the second zipper locks the two sides is the offset or slip. The time it takes for the slip to occur is the rise time. The slip divided by the rise time is slip rate, that is how fast the slip occurs on the fault.
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23. The movement of the ground is three dimensional and can be completely described by examining it on three perpendicular axes. The natural choices are along the vertical and two horizontal directions. We have chosen the headings of 118° and 28°, parallel and perpendicular to the San Andreas, respectively, for positive horizontal ground velocity.
24. Y. Hisada *et al.*, in *Proc. Fourth Int. Conf. Seismic Zonation*, Stanford, CA (1991), vol. II, p. 245; A. Edelman and F. Vernon, unpublished report.
25. The amplitude of seismic waves can be attenuated by many different mechanisms, but the primary mechanisms are geometrical spreading and intrinsic attenuation. Geometrical spreading of the wavefront attenuates the amplitude as a direct result of conservation of energy. As the wavefront expands with distance from the source, the energy is spread over a greater surface area resulting in a smaller energy per unit area and accordingly a smaller amplitude. A simple approximation to geometrical spreading for body waves is: $A \propto 1/R$, where A is the amplitude of the wave and R is the distance between the source and the observer. For surface waves (Love and Rayleigh) the geometrical attenuation is approximately: $A \propto 1/\sqrt{R}$ so that surface waves decay more slowly than body waves. Intrinsic attenuation is the irreversible loss of wave energy due to internal friction as the waves propagate through the medium. The amplitude decay due to internal friction can be approximated by : $A \propto \exp(-\pi ft/Q)$ where f is frequency, t is the time it takes for a wave to travel from the source to the observer, and Q is a measure of the energy loss due to internal friction. We have omitted intrinsic attenuation in our simulation. However, comparison of observed seismic data from the 17 January 1994 **M** 6.7 Northridge event to an elastic simulation of the earthquake (*3*) suggests that intrinsic attenuation has limited effects on ground motion for frequencies less than 0.4 Hz, as used in this study.
26. The rupture directivity is an effect that modifies the energy in seismic waves depending on the angle between the observation point and the direction of rupture propagation [A. Ben-Menahem, *Bull. Seismol. Soc. Am.* **51**, 401 (1961)]. Directivity increases the energy in the direction the rupture propagates and decreases the energy in the back direction. A seismic pulse in the forward direction increases in amplitude and contracts in time; a seismic pulse in the back direction decreases in amplitude and expands in time. The directivity effect combined with the radiation pattern of S waves leads to large velocity pulses (on the order of 1.0 m/s) in the forward direction of earthquake ruptures [R. Archuleta and S. Hartzell, *Bull. Seismol. Soc. Am.* **71**, 939 (1981); R. Archuleta, *J. Geophys. Res.* **89**, 4559 (1984); (*4*)].
27. The cumulative kinetic energy for each gridpoint (x, y) on the surface is given by:

$$E_k(x,y) = \frac{1}{2}\rho(x,y)\int_0^T \dot{u}^2(x,y,t)dt$$

where k is the component of motion, ρ is the density, $\dot{u}(x,y,t)$ is the velocity seismogram, and T is the duration of the seismogram. The cumulative energy includes the amplitude and duration of the signal. The total energy is the sum of all three components: $E_{tot}(x,y) = E_{118}(x,y) + E_{28}(x,y) + E_v(x,y)$.
28. There are formulas for relating the height of structures with the period of the lowest-mode response. For example, with $h(ft) = (period/0.035)^{1.333}$ [*Uniform Building Code* (International Conference of Building Officials, Whittier, CA, 1988)], a 3.0-s period corresponds to a 115-m-high steel frame building. Such formulas serve as a guide; more flexible buildings or buildings made of different materials may be significantly shorter for a given period. For example, the Sherman Oaks building has a lowest-mode period of 3 s (0.33 Hz) but is 50.6 m high (13 stories) [Chapter 2, *Earthq. Spectra Suppl. C* **11**, 13 (1995)].
29. The classification of soils and rock in geotechnical engineering depends on the S wave velocity. A firm to hard rock has an average shear-wave speed greater than 700 m/s [R. Borcherdt, *Earthq. Spectra* **10**, 617 (1994)]. The lowest velocity in our model is 1.0 km/s (Table 2).
30. Many papers document the amplification of seismic waves due to local near-surface geology [for example, K. Aki, *Am. Soc. Civil Eng. Proc. Spec. Conf. Earthq. Eng. Soil Dyna.* **2**, 1 (1988)].

31. We are grateful to MIT's Earth Resources Laboratory for permission to use the nCUBE 2 parallel computer. We thank H. Magistrale for allowing us to use his 3D LA basin model and S. Day for advice on insertion of the double-couple source into the finite-difference scheme. This work was supported by the Southern California Earthquake Center (SCEC), USC 572726 through the NSF cooperative agreement EAR-8920136.

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Quantum Computers, Factoring, and Decoherence

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It is known that quantum computers can dramatically speed up the task of finding factors of large numbers, a problem of practical significance for cryptographic applications. Factors of an L-digit number can be found in $\sim L^2$ time [compared to $\sim\exp(L^{1/3})$ time] by a quantum computer, which simultaneously follows all paths corresponding to distinct classical inputs, obtaining the solution from the coherent quantum interference of the alternatives. Here it is shown how the decoherence process degrades the interference pattern that emerges from the quantum factoring algorithm. For a quantum computer performing logical operations, an exponential decay of quantum coherence is inevitable. However, even in the presence of exponential decoherence, quantum computation can be useful as long as a sufficiently low decoherence rate can be achieved to allow meaningful results to be extracted from the calculation.

The uniqueness of the prime factorization of a positive integer is the Fundamental Theorem of Arithmetic (*1*). In practice, the determination of the prime factors of a given number can be an exceedingly difficult problem, even though verification is trivial. This asymmetry is the basis for modern cryptography and provides secret codes used not only on your own bank card but also to transfer diplomatic messages between embassies.

Attempts to undermine the security provided by the difficulty of factorization have by and large met with failure, even with the aid of powerful modern computers. In fact, this problem is widely believed to have no polynomial-time solution (*2*), although a proof of this statement has remained elusive. The best known classical computer algorithm (*3*) for factoring a number N of L digits takes a time exponential in $L^{1/3}$. In contrast, Shor (*4*) has shown recently that with the help of a quantum computer, one can factor numbers in a random polynomial amount of time. Therefore, such computers could be a threat to what is presently one of the most common methods of encryption. However, it is still unknown whether such machines are practical, because they depend crucially on quantum-mechanical behavior that is uncommon to our mostly classical world (*5*).

The quantum factoring algorithm uses in an essential way the coherence of a quantum wave function. To factor a number N, one chooses a number x at random and calculates its order, r, modulo N, that is, one finds r such that $x^r \equiv 1 \bmod N$. Once r is known, factors of N may likely be found by computing the greatest common divisor of $x^{r/2} \pm 1$ and N (*1*, *4*). The difficulty is in calculating r, which is solved by the quantum factoring algorithm: First choose a smooth number (one with small prime factors) q such that $N^2 < q < 2N^2$ and build the state

$$|\psi_1\rangle = \frac{1}{\sqrt{q}} \sum_{a=0}^{q-1} |a,0\rangle \qquad (1)$$

from which can be obtained (with a quantum computer) (*6*)

$$|\psi_2\rangle = \frac{1}{\sqrt{q}} \sum_{a=0}^{q-1} |a, x^a \bmod N\rangle \qquad (2)$$

We can now Fourier transform this pure state (again, with a quantum computer) to get

$$|\psi_3\rangle = \frac{1}{\sqrt{q}} \sum_{m=0}^{q-1} \sum_{a=0}^{q-1} e^{i2\pi am/q} |m, x^a \bmod N\rangle \qquad (3)$$

and can measure both arguments of this superposition, obtaining the value c for m in the first argument and some x^k as the answer for the second one (k being any number between 0 and r). Given the pure state $|\psi_3\rangle$, probabilities of different results for this measurement will be given by the probability distribution

$$P(c,x^k) = \left| \frac{1}{q} \sum_{a=0}^{q-1}{}' e^{i2\pi ac/q} \right|^2 \qquad (4)$$

where the prime indicates a restricted sum over values of a that satisfy $x^a \equiv x^k \bmod N$. Independent of k, $P(c, x^k)$ is periodic in c with period q/r, but because we know q, we can determine r with a few trial executions [an example of $P(c, x^k)$ is shown in Fig. 1]. A measurement thus gives with high probability $c = \lambda q/r$, where λ is an integer that corresponds to a particular peak in Fig. 1. With a few runs of the program, we can deduce r and thus the factors of N.

This algorithm assumes that the quantum computer is completely isolated. In practice this will certainly not be the case. It is the effect of imperfect isolation that we study here. An obvious effect is that the quantum computer will lose energy. This happens at the rate τ_{rel}^{-1}, the inverse of the relaxation time scale. It is relatively easy to make systems for which τ_{rel} can be very large and thus allow for a reasonable number of operations. A much more insidious effect of imperfect isolation is decoherence (*7*). Decoherence is caused by continuous interaction between the system (in our case, the quantum computer) and the environment (*7*, *8*). As a result, the state of the environment "monitors," and therefore becomes correlated with, the state of the computer. As a quantum system evolves, information about its states leaks out into the environment, causing the states to loose their purity and, consequently, their ability to interfere.

It is important to realize that the time scale for decoherence τ_{dec} is usually much smaller than the one for relaxation. For example, an oscillator of mass m in a superposition of coherent states (separated by a distance Δx from each other) interacting linearly with a bath at temperature T has the decoherence time (*9*)

$$\tau_{dec} \sim \tau_{rel} \left(\frac{\lambda_{dB}}{\Delta x} \right)^2 \qquad (5)$$

where λ_{dB} is the thermal de Broglie wavelength. This expression is valid for high temperatures only; at low temperatures, τ_{dec} becomes inversely proportional to the cutoff frequency of the bath. No net energy transfer is needed to effect decoherence. This implies a much greater sensitivity of quantum computation to decoherence than to the relaxation process.

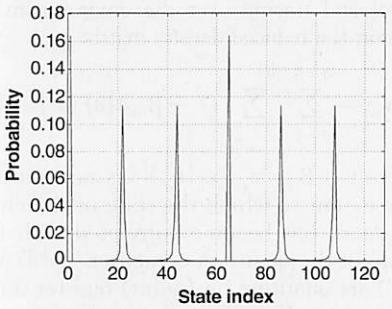

Fig. 1. Probability distribution for the measurement of c in the state given in Eq. 3 with $N = 21$, $q = 128$, $x = 5$, and $k = 3$. The broadening of the peaks is from the use of a discrete Fourier transform with q possible modes; a continuous Fourier transform would have given delta functions.

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The decoherence process has been proposed as a mechanism for enforcing classical behavior in the macroscopic realm. Decoherence results in environment-induced superselection (7– 9), which destroys superpositions between the states of a preferred pointer basis (a set of states selected by the interaction with the environment) (8). Classical computers are already decohered: computation takes them through a predictable sequence of such pointer states, which are stable in spite of the environment; thus, classical computers cannot be put in arbitrary superpositions. However, coupling with the environment will be inevitable for any system that implements quantum computation. Here we show the effect of decoherence on the interference pattern produced as a result of executing the quantum factoring algorithm.

Our model involves the introduction of the environment as a system external to the computer; its state is represented by third label. The input state may thus be written as

$$|\tilde{\psi}_1\rangle = \frac{1}{\sqrt{q}} \sum_{a=0}^{q-1} |a, 0\rangle \otimes |\varepsilon \qquad (6)$$

where ε is the initial state of the environment and $\otimes$ is the direct product of vector spaces. The environment is initially uncorrelated with the computer; however, it is likely that the bits necessary for the calculation of $x^a \bmod N$ will interact with the environment, so that the next state

$$|\tilde{\psi}_2\rangle = \frac{1}{\sqrt{q}} \sum_{a=0}^{q-1} |a, x^a \bmod N\rangle \otimes |\varepsilon_a\rangle \qquad (7)$$

leaves the environment partially correlated with the state of the computer. When the physical representation for the computer's quantum bits is diagonal in the pointer basis of the environment, decoherence results in no adverse effects when measuring the second label of $|\tilde{\psi}_2\rangle$. Such a design would be optimal. We thus focus on the effects of decoherence on the first label by suppressing the second label and tracing over the environment to obtain the reduced density matrix

$$\rho_{\text{red}} = \sum_{a=0}^{q-1}{}' \sum_{a'=0}^{q-1}{}' (1 - \beta_{aa'}) |a\rangle \langle a'| \qquad (8)$$

Here $1 - \beta_{aa'} = |\langle \varepsilon_a | \varepsilon_{a'} \rangle|^2$ is a measure of the extent to which the state of the environment has become correlated with the state of the quantum computer. If $|a\rangle$ and $|a'\rangle$ are quantum bit (qubit) register states diagonal in the pointer basis, then we may take

$$1 - \beta_{aa} \approx \exp[-\xi \cdot (a \text{ XOR } a')] \qquad (9)$$

where the exclusive-or (XOR) function gives the Hamming distance (*10*) between a and a'. Decoherence is characterized by a parameter ξ, which depends on the particular realization of the quantum computer. The measurement results in a probability distribution, shown in Fig. 2, which differs from the one in Eq. 4 (see Fig. 1) in that non-zero-probabilities have appeared between the peaks and that these peaks have decreased in amplitude. Note that decoherence does not increase the width of the peaks.

The qualitative effect of decoherence is well approximated by the simpler function $1 - \beta_{aa} \equiv 1 - \beta(1 - \delta_{aa'})$, where β is a constant. For $\beta = 0$ we get the state with complete coherence, and for $\beta = 1$ one with complete decoherence (that is, a matrix diagonal in the pointer states). In the limit of $\beta \sim 0$, we may understand β as the fractional amount of information lost to the environment. For $\beta \approx 0.5$, the probability between the peaks (Fig. 2) is equal to one of the peaks, and thus there is as much chance to get a correct answer as a wrong one. Let $L = \log N$. Once $(1 - \beta)^{-1} \sim O[\exp(L^{1/3})]$, the quantum computer becomes as inefficient as a classical one, requiring a number of trials exponential in $L^{1/3}$ to factor N. Note that β is analogous to the ratio between the amplitude of the destructive and constructive interference in the double-slit experiment, also called the fringe visibility function.

When we assume that the effect of the environment has a Markoffian character, then $\beta \approx 1 - e^{-n_{\text{op}}\alpha M} \approx n_{\text{op}}\alpha M$, where α is the coherence lost per bit in a single logic operation, M is the number of memory qubits involved, and n_{op} is the number of time steps required to complete the computation. To factor a number $N \sim e^L$, the quantum algorithm requires $M \sim L$ and $n_{\text{op}} \sim L^2$. With perfect operation, each execution gives a factor with probability $O(1/L)$, and thus with decoherence, the required number of trials is $O(L/(1 - \beta))$. When this is expressed in terms of α and L, the number of trials required find a factor of N is of order

$$\text{Number of trials} \sim L\exp(L^3\alpha) \qquad (10)$$

To give performance better than the classical algorithm, we must therefore have

$$\alpha < L^{-8/3} \qquad (11)$$

Designs for quantum computers have been suggested (*11– 14*) and some possible difficulties investigated (*5*, *15*). Common to these designs is the model of a simple two-state system (qubit) interacting with an ensemble of oscillators (the environment), from which we can get an idea of what α is. Such an analysis, with the well-studied spin-boson Hamiltonian (*16*), straightforwardly gives the result that in a typical time step of a quantum computer, the effect of coupling to a zero-temperature environment is to decrease the off-diagonal term of the density matrix by the amount (*17*)

$$\alpha \approx \frac{\varepsilon^2 \eta}{2\pi}\left[O(1) - \log\left(\frac{\Delta}{\Lambda}\right)\right] \approx \frac{\gamma}{\Delta} \qquad (12)$$

where ε is the (dimensionless) coupling strength, η is the resulting viscosity on the qubit (which will determine the rate of the loss of energy), Λ is a high-frequency cut-off of the bath, Δ^{-1} defines the time scale of a single operation, and γ is coherence lost per unit time. For example, in the experimental realization of an ion-trap quantum logic gate by Monroe *et al.* (*18*), $\gamma \approx 10^3$ Hz and $\Delta \approx 10^4$ Hz. This gives $\alpha \approx 0.1$, which implies that a quantum computer would outperform a classical one only for a number no more than a few bits in length. On the other hand, the original proposal of Cirac and Zoller (*14*) assumes that the ultimate source of errors will be spontaneous emission and estimates that $\gamma \approx 0.1$ Hz and $\Delta \approx 10^5$ Hz, which naïvely gives $\alpha \approx 10^{-6}$. This value would allow factoring of a number of perhaps a few hundred bits. Although the latter estimate is promising, we stress that it may be overly optimistic, because γ does not reflect all of the decoherence processes that may be taking place.

Unruh has also analyzed the impact of decoherence on quantum computation (*15*). He computed the behavior of a static memory, which exhibited three regimes of the decay of coherence of a qubit [an early one depending on the state of the qubit; a "quantum" regime where $\beta \sim 1/(\lambda t)$; and a "thermal" regime that starts at $\hbar/k_B T$ (k_B, Boltzmann's constant)] in which $\beta \sim \exp(-\varepsilon^2 Tt)$, and concluded that the time taken by the quantum computer to complete the calculation must be smaller than the (thermal) time scale for which the decoherence (β) becomes exponential. We differ with these conclusions on two counts.

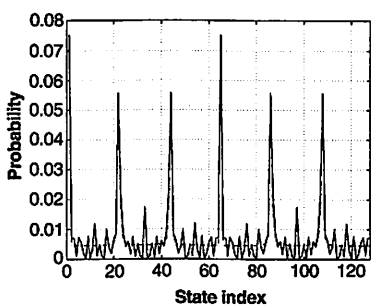

Fig. 2. Effect of decoherence on the probability distribution for the value of c. The state is given by Eq. 8 once a is Fourier transformed. The decoherence parameter (Eq. 9) has been taken to be $\xi = 0.1$. Our constant-beta approximation, with $\beta = 0.58$ (dash-dotted line), shows good agreement.

(i) When the computer is carrying out operations (rather than just trying to remember some state), the decay of quantum coherence is inevitably exponential even in the limit of zero temperature. Nevertheless, (ii) exponential decay is not a reason to give up: It is the rate of that decay that ultimately matters. This rate is given by Eq. 12 in the case studied here, and by $\varepsilon^2 k_B T/\hbar\Delta$ for the temperature-dominated regime. In either case, by increasing isolation (that is, by making ε small) and making the clock rate Δ large, one can take the computation well beyond the time scale (thermal or otherwise) when the loss of coherence becomes exponential with time.

Ultimately, the savior of general-purpose quantum computing lies in the success of quantum error correction. It is generally true that the discrepancy between the correct state of a quantum computer and the actual state will initially increase only quadratically in time (t)

$$|\langle\psi_{\text{actual}}|\psi_{\text{ideal}}\rangle|^2 \simeq 1 - (\delta t)^2 \qquad (13)$$

where δ is the variance of the difference between the ideal and actual energy. Thus, the "watchdog effect" can be used to stabilize the computation (*19*), for when the computer is measured often enough, on a time scale t/ν that is short compared with $1/\delta$, it will stray from the correct evolution only a little, so that the probability of being correct is

$$|\langle\psi_{\text{actual}}|\psi_{\text{ideal}}\rangle|^2 \simeq \left[1 - \left(\frac{\delta t}{\nu}\right)^2\right]^\nu \qquad (14)$$

which can be much closer to unity than Eq. 13. Performing a measurement on a qubit at the instants when it is expected to be in the eigenstate of the measured observable with certainty according to $|\psi_{\text{ideal}}\rangle$ will project the actual state of the computer into a state closer to $|\psi_{\text{ideal}}\rangle$ and may thus offer a way of implementing such a "watchdog stabilization" (*20*). Although this effect is useless once the loss of coherence becomes exponential (as it is for spontaneous emission), such a scheme may be helpful in keeping at bay errors from timing inaccuracies and environmental differences. For example, in the linear ion trap computer (*14*), both the center-of-mass phonon and the auxiliary levels of the ions have predictable occupation numbers at well defined instants during ideal operation. This promise, coupled with the results obtained from our analysis of the impact of decoherence on the quantum factoring algorithm, bring some hope to the eventual reality of quantum computers and motivate further experimental investigations in this field.

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Correlated Variations in the Solar Neutrino Flux and the Solar Wind and the Relation to the Solar Neutrino Problem

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Solar wind parameters from the Massachusetts Institute of Technology (MIT) plasma experiment on the IMP 8 spacecraft overlap ~19 years of published neutrino flux observations from the Homestake experiment. A strong correlation is found between neutrino flux and solar wind properties, in particular, the solar wind mass flux. The correlation is significantly better than any anticorrelation with sunspot number and is comparable to those previously found with photospheric magnetic flux and shifts in *p*-mode frequencies. If current notions of solar structure are correct, these observations require new fundamental physics of neutrinos. For a proper choice of neutrino parameters, the level of variations is consistent with resonant conversion of electron neutrinos to a nondetected flavor eigenstate mediated by the magnetic field in the sun's convective zone. The solar wind mass flux may act as a proxy for this field, producing the solar wind–neutrino flux connection.

The measured average neutrino flux from the sun is low compared with predictions based on solar models. This discrepancy, a factor of more than 3, originally showed up in the data of the ^{37}Cl experiment in the Homestake Gold Mine in South Dakota (*1*) and is known as the solar neutrino problem (*2*). Low neutrino fluxes have been confirmed for neutrino energies other than those measured at Homestake by the Kamiokande-II water Cherenkov experiment (*3*) and the SAGE (*4*) and GALLEX (*5*) gallium experiments.

The Homestake rate appears to exhibit a time-variable component. A possible anticorrelation with solar activity has been studied by a number of investigators. The correlation has remained suspect because of the low counting statistics, questionable correlation, and difficulty of explanation (*6–8*). Confirmation from the other solar neutrino experiments now operating is problematic because of the shorter times the experiments have been running and large statistical uncertainties.

Strong (time-dependent) correlations have been reported between shifts in solar *p*-mode frequencies and the Homestake capture rate (*9*, *10*). Recently an anticorrelation of capture rate with photospheric magnetic flux that is stronger than that with sunspot number has been found (*11*); this anticorrelation increases as flux away from the center of the solar disk is excluded. In this report I show that there is a corresponding large correlation between capture rate and the solar wind flux as measured near Earth by the MIT plasma experiment on the IMP 8 satellite.

The solar wind data used here are from the MIT Faraday cup plasma analyzer (*12*). The only significant data gap is from part of 1982 to 1983, resulting from a problem with

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the instrument-mode setting during that period. The relative calibration of the MIT experiment has remained extremely stable over the years since launch; with the use of continuous data from one instrument on one spacecraft, issues of intercalibration with other measurements were avoided (*13*).

Data acquired in the solar wind were averaged over the periods during which neutrino capture fluxes were monitored for a given run. For composite variables such as the solar wind proton flux, the product of the density and speed was formed from the hourly averaged density and hourly averaged speed, and these were then averaged over the given Homestake run. In all, 62,999 hourly averages were formed into 91 bins, which overlap with neutrino capture runs 31 through 126 (*14*). These averages were used for comparison with the neutrino capture flux data. There are no IMP 8 data during Homestake run 76 because of the instrument problem referred to above (*15*).

The same technique was applied to daily values of the sunspot number for the same time period (*16*). In all, 6796 values of the daily sunspot number were averaged into 92 intervals. These correspond to the same bins used for the solar wind parameters, with the exception that the data for Homestake run 76 was left in the sunspot data set.

Neutrinos were presumed to have sufficiently small masses so that they are relativistic and reach Earth from the sun in ~8 min. Hence, the density and magnetic structure of both the solar wind and the sun through which a given neutrino travels are essentially constant in time during the propagation. The data accumulation intervals for the neutrino runs were also long compared with the solar wind propagation time to Earth (~4 days) and effectively average over the 27-day solar rotation period and subsolar heliolatitude range (±7°15′) as viewed from Earth. Given the typical persistence of solar wind structures over several solar rotations, correlated variations tend to be reinforced in the time periods of the individual Homestake runs; such persistence can also be amplified in the data by averaging.

Use of a boxcar average of the data points (a low-pass filter) improved the apparent significance of the correlations (*6*, *9*, *11*, *17*). Sunspot data and ^{37}Ar capture data are shown in Fig. 1 as a function of time with 3-point sliding boxcar averages. The neutrino capture rate is scaled to the sunspot number from a simple linear regression using the two filtered data sets. The amplitude of the neutrino capture rate variations is significantly reduced in comparison with that of the sunspot number variations. The scaling is from a linear regression with

$$y_\nu = ax_\nu + b \qquad (1)$$

with $a \approx -53.1$ and $b \approx 116$. Here, x_ν are the 3-point sliding averages of the measured neutrino capture rate (equal to the production rate of ^{37}Ar), and y_ν are the scaled values that are plotted in the figure with a and b derived from the linear regression (*18*).

The solar wind (proton) flux and neutrino capture rate are plotted in Fig. 2 with the same format and methodology used for Fig. 1. The scaling of the neutrino flux is given with Eq. 1 and $a \approx 1.15 \times 10^8$ cm^{-2} s^{-1} and $b \approx 3.31 \times 10^8$ cm^{-2} s^{-1}. The tracking of the two variations is much more striking in Fig. 2 than in Fig. 1.

Linear regression analyses were also performed on other solar wind quantities. The largest value of the linear regression coefficient (Pearson's r) occurs for the solar wind particle flux ($r = 0.419$), although it is large for any of the solar wind moments (r = 0.371, 0.375, and 0.354 for the density, momentum flux, and product of density and thermal speed, respectively). Of the quantities examined, only the speed and thermal speed are less well correlated (r = 0.0843 and 0.0438, respectively) than the sunspot number ($r = -0.183$; a negative value being an anticorrelation).

This analysis has also been applied to the single data points and 5-point sliding averages. The correlations in the case of the 3-point averages are always better than those from just the individual data points themselves (Table 1). Formal significance levels are extremely small for the sliding averages, but the low-pass filtering (by means of the sliding average) introduces additional correlations between points, reducing the true number of degrees of freedom. For the solar wind mass flux, the 3-point sliding average r and 30 points yield a significance P of 0.021; the 5-point regression and 18 effective points yield a significance of 0.088.

Reported correlations with emergent magnetic flux (*11*) used 5-point running averages over the period 1970.3 through 1991.6. Excluding regions with heliographic latitudes in excess of 5°, the Spearman correlation coefficient was −43%, not quite as large (in magnitude) as values obtained with the solar wind mass flux correlation for both 3- and 5-point sliding averages (Table 2). Comparable correlations and significance levels with respect to other solar phenomena have also relied on similar methodology (*10*).

As a check on the results, nonparametric tests were applied to the solar wind flux and sunspot number (Table 2) for each averaging interval (again 1-, 3-, and 5-point sliding averages in the time series). These nonparametric analyses confirm the linear re-

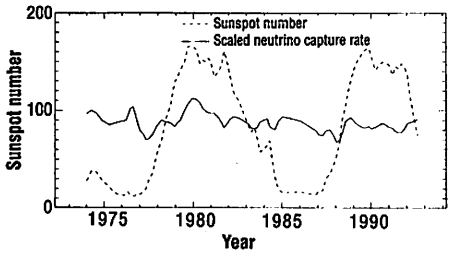

Fig. 1. Sunspot number and neutrino capture rate (^{37}Ar capture data) as a function of calendar year. The period covered starts with the beginning of the IMP 8 mission and goes through the end of the published neutrino capture data. The neutrino capture rate has been scaled to the sunspot number average in the neutrino-run bins based on a simple linear regression, which is based on 3-point sliding averages of the binned data (Eq. 1). Note that the neutrino capture rate is inverted because the correlation coefficient is negative. The end points not averaged over have been suppressed.

Table 1. Linear regression results. Averages are sliding boxcar averages of both binned data sets: 92 bins are used for the sunspot data (based on daily sunspot number) and 91 bins are used for solar wind data (based on hourly averages of proton density and proton speed). Probabilities P are two-sided significance levels for the acceptance of the null hypothesis (that is, that the data sets are uncorrelated). The z is from Fisher's z transformation and can be used to compare the significance levels (*18*).

Average	Sunspots			Particle flux		
	r	z	P	r	z	P
1-point	−0.143	−0.144	0.174	0.202	0.205	0.0543
3-point	−0.183	−0.186	0.0800	0.419	0.446	3.60×10^{-5}
5-point	−0.213	−0.217	0.0411	0.414	0.440	4.52×10^{-5}

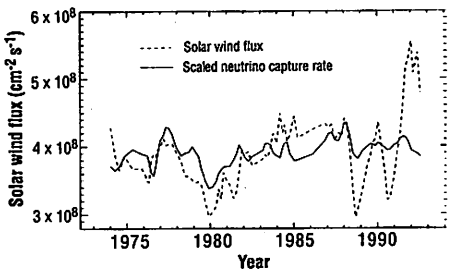

Fig. 2. Solar wind proton flux and neutrino capture rate as a function of calendar year. The neutrino capture rate is scaled to the solar wind flux with linear regression parameters from 3-point sliding averages of the binned data.

sults: lack of correlation (the null hypothesis) between the solar wind flux and the neutrino capture rate is very unlikely (less than 3%) with the individual 91 data points. This probability compares with less than 17% for a sunspot correlation.

Since the early 1960s, the density, velocity, and temperature of the solar wind near Earth orbit have been monitored with varying degrees of regularity. Long-term variations in these quantities are difficult to monitor because of (i) the small amplitudes of long-term variations, (ii) large short-term variations, (iii) incomplete data coverage, and (iv) inherent difficulties in intercalibrating different instruments on different satellites (*19*). Over the long term, the solar wind mass flux does not vary greatly, consistent with the results reported here. Mass flux is known to vary with solar wind speed, and the distribution of wind speed in heliographic latitude and longitude varies with the phase of the solar cycle (*20*). Hence, some variation of the mass flux as seen at Earth can be ascribed to the excursion of Earth (and therefore IMP 8) in heliographic latitude.

The near constancy of the solar wind mass flux observed near the ecliptic plane is linked to coronal dynamics that couple to solar magnetic field structure at lower levels. Variations are the result of varying expansion factors in magnetically open regions (*21*): the larger the magnetically open regions, the less the areal expansion and the larger the mass flux. Although the mass flux and coronal-hole characteristics and filling factors vary with solar activity, a clear-cut oscillatory behavior in the these quantities is not present. Hence, a correlation between solar wind properties and the neutrino capture flux, both of which exhibit aperiodic behavior, appears all the more remarkable.

A consistent picture is emerging in which increased neutrino flux corresponds to (i) decreased line-of-sight magnetic flux (*11*), (ii) decreased *p*-mode frequencies (*9*, *10*), (iii) decreased sunspot number (*6*, *7*), (iv) increased solar radius (*10*), and (v) increased proton and mass flux in the solar wind (this work). Internal consistencies exist in this list as well: (i) and (ii) (*22*), (ii) and (iv) (*10*), (ii) and (iii) (*23*), and (i) and (iii) (*11*) are each consistent.

The model of (*24*) suggests that the average radial component of the photospheric magnetic field B_r decreases with *p*-mode frequencies as observed (*11*). In spherically symmetric solar wind models, the ratio of mass flux to radial magnetic-field component is a field-line constant, so larger B_r implies larger flux. However, this is a local argument and does not take closed fields into account. Observations show that the mass flux at the coronal base tends to increase linearly with the (radial) magnetic field there. Similarly, the logarithm of the mass flux there tends to increase roughly quadratically with the flux-tube expansion factor (*25*). These combined effects result in a larger photospheric magnetic field correlated with a smaller magnetic flux at Earth, that is, less (net) radial flux (fewer closed regions) should correspond to lower areal expansion factors (*21*) and larger solar wind flux, as observed. All of these observed variations tend to decrease if larger averaging intervals are used.

The correlation study with photospheric magnetic flux supports the hypothesis that the neutrinos interact with magnetic fields along their flight paths (*11*). This hypothesis is also supported by observations of the solar wind mass flux at Venus. Measurements are available from the plasma analyzer on the Pioneer Venus Orbiter (PVO) spacecraft from orbit insertion in 1978 through orbit decay in 1992 (Fig. 3).

The PVO data are offset from, but track, the IMP 8 data from the beginning of 1979 through 1985. From 1985 through the beginning of 1988, the PVO mass flux differs dramatically from both the IMP 8 flux and scaled neutrino count rate. During this period, PVO (and Venus) were immersed in a low-speed, high-density solar wind usually associated with the equatorial streamer belt, whereas IMP 8 (and Earth) spent most of this period at higher heliolatitudes and observed different wind properties (*26*) (the excursion of Venus from the solar equator is less than that of the Earth, only ~4°). Both

Table 2. Nonparametric statistical tests for solar wind flux and sunspots. The same data are used as in Table 1. Here, $r_{Spearman}$ is Spearman's rank correlation coefficient; σ_D, the variance of sum squared difference of ranks; τ, Kendall's τ; σ_τ, the variance in the τ statistic; and P_{rs}, P_D, and P_τ, the corresponding significance levels for these statistics (*18*).

Average	$r_{Spearman}$	P_{rs}	σ_D	P_D	τ	σ_τ	P_τ
				Solar wind flux			
1-point	0.233	0.0263	−2.21	0.0269	0.155	2.17	0.0297
3-point	0.440	1.29×10^{-5}	−4.17	3.00×10^{-5}	0.308	4.33	1.50×10^{-5}
5-point	0.450	7.4×10^{-6}	−4.27	1.92×10^{-5}	0.323	4.53	5.8×10^{-6}
				Sunspots			
1-point	−0.144	0.170	1.37	0.170	−0.112	−1.58	0.113
3-point	−0.0801	0.448	0.763	0.446	−0.0588	−0.830	0.406
5-point	−0.126	0.232	1.20	0.223	−0.0855	−1.21	0.227

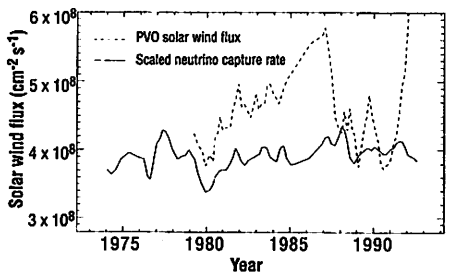

Fig. 3. Solar wind proton flux at Venus as monitored by the plasma analyzer on board PVO. The scales and neutrino capture rate are duplicated from Fig. 2. The PVO data overlap Homestake runs 57 through 125; 66 runs actually overlap. Solar wind parameters were obtained from Ames Research Center (by means of the World Wide Web) and processed in the same manner as the IMP 8 data; the mass flux is decreased by a factor of $(0.72)^2$ to normalize the data to 1 AU. The PVO fluxes are generally higher than those from IMP 8 because of unmodeled instrument offsets.

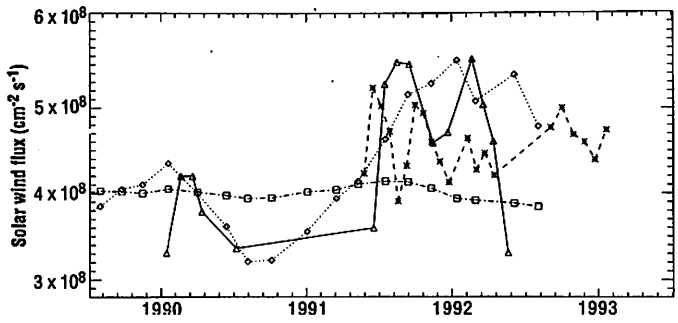

Fig. 4. Comparison of relative count rates from the gallium experiments [GALLEX (*) and SAGE (△)] with solar wind flux variations (◇). The same conversion to mass flux as found from the Homestake data (□) is applied but reduced by an arbitrary factor of 80; error bars on each measurement are comparable to the measurements themselves. All data are shown as 3-point sliding averages (see Fig. 2). The SAGE results include 15 runs from January 1990 through May 1992 (*4*). There is no reported statistical evidence for variations in the background; the average rate is $73 \pm^{18}_{16}$ (statistical) $\pm^{5}_{7}$ (systematic) solar neutrino units (1 SNU = 10^{-36} captures per target atom per second). GALLEX results include 21 runs made from 14 May 1991 through 3 February 1993 (*5*). Combined analysis for all runs is 87 ± 14 (statistical) ± 7 (systematic) SNU. More recent results are 79 ± 12 SNU; the standard model prediction for gallium experiments is 132 ± 7 SNU (*2*).

low-speed flows and high-speed streams are nonradial near the sun (*20*) and are not necessarily representative of the region traversed along a radial path by the neutrinos. Such flow and solar activity not linked to the neutrino modulation mechanism could explain the relatively worse correlation between solar wind and neutrino fluxes in Figs. 2 and 3 from ~1988 through the end of the data in 1992 (part of the increase at the end of 1991 is an artifact associated with the end of the PVO mission before entry into the atmosphere of Venus).

The SAGE data runs (Fig. 4) have no counting events during times of low solar wind flux. High rates are found during the summer of 1991 (and in Homestake run 117), coincident with major solar activity that produced radio emissions from the outer heliosphere, although this is also the period that the correlation of the Homestake data with the solar wind mass flux is not as good as for previous periods. However, the peak rates in the radiochemical experiments occur in nominally nonoverlapping runs. Furthermore, similar solar activity levels in mid-1982 through early 1983 produced no correspondingly anomalous rates in Homestake runs at corresponding times (runs 75 and 76) (*27*). The relatively few data points and large counting and systematic errors make identification of temporal variations problematic. No consistent pattern of time variation is suggested by either the peak values or the current series of gallium detector data. There may be a suggestion of a correlation between the IMP 8 and SAGE data, with some lag in the solar wind data. There has been no significant time variation reported in the Kamiokande-II experiment (*3*).

Proposed solutions to the solar neutrino problem can be classified as astrophysical or nonastrophysical. Astrophysical solutions posit that the production rate of the neutrinos is not calculated correctly because of some fundamental flaw in our understanding of how the sun produces energy. Suppressed or fluctuating neutrino fluxes result from conditions in the solar core (*6*). However, these solutions are not consistent with detailed results from helioseismology (*28*). Nonastrophysical solutions suppose that the production rate of the neutrinos is calculated correctly (*8*), that there is no link between the solar core and (short-term) photospheric variations, and that the suppressed fluxes monitored at Earth are caused by neutrino physics outside of the standard model of electroweak interactions (*8*, *28*, *29*).

The variations in *p*-mode splittings, emergent magnetic flux, and solar wind flux with the neutrino capture flux suggest a connection between solar activity and extended neutrino physics in the convective zone of the sun (*30*). The observed time variations do not support other nonastrophysical solutions (*8*).

Although a magnetic moment is acquired by massive Dirac neutrinos, it is too small to couple effectively to the solar magnetic field. A further extension of electroweak theory can provide sufficiently large magnetic moments $\mu_\nu \sim 10^{-10}\mu_{Bohr}$ (*30*) (where μ_{Bohr} is the Bohr magneton), although such large values may violate limits derived from neutrino observations of supernova SN 1987A (*31*). Energy and time dependencies are coupled to this solution if neutrinos are massive and if the spin-flip is moderated by a resonance similar to that in the MSW (Mikheyev-Smirnov-Wolfenstein) effect (*32*).

If the mass difference parameter $\Delta_0 \equiv m_2^2 - m_1^2 > 0$ is sufficiently small for convection zone resonance (*32*, *33*), then all of the fusion neutrinos are born well above their resonant density. The survival probability of electron neutrinos (ν_e) can be estimated as (*32*)

$$P(\nu_e \rightarrow \nu_e) \approx \frac{1}{2} - \frac{\frac{1}{2} - e^{-\pi^2 r_0 L_{osc}/L_B^2 \cos 2\theta}}{\sqrt{1 + L_{osc}^2/L_{B_0}^2 \cos^2 2\theta}} \quad (2)$$

where the argument of the exponential is evaluated at the resonant density (*34*). For a spatially varying magnetic field, the effective vacuum value B_0 is less than the resonant value B_{res} and should be about the same for all neutrinos, whereas B_{res} varies with energy because the magnetic field, a function of location, varies with the resonant density.

As an example (Fig. 5), for $\Delta_0 \cos(2\theta) \sim 10^{-8}$ eV^2 and $\mu_\nu \sim 1.5 \times 10^{-10}$ μ_{Bohr}, plausible levels and variations in the survival probability can be reproduced. All neutrinos pass through an MSW resonance as well (*32*), but the resonance is nonadiabatic and has no effect. Intermediate energy neutrinos are both suppressed and modulated the most (*2*); low-energy neutrinos (*4*, *5*) are not suppressed as much because most of them pass through corresponding resonances in the radiative zone (*32*), where the magnetic field strength is small. Small penetrations of large field strengths to lower levels could produce large excursions in neutrino flux. Less time variation occurs in the higher energy neutrinos (*3*). Measured suppression levels can be explained by detection of the converted neutrinos but with less efficiency (*33*). The solar wind mass flux is a proxy for the magnetic field strength in the convection zone (Eq. 2) and provides the physical link between the solar wind and neutrino fluxes.

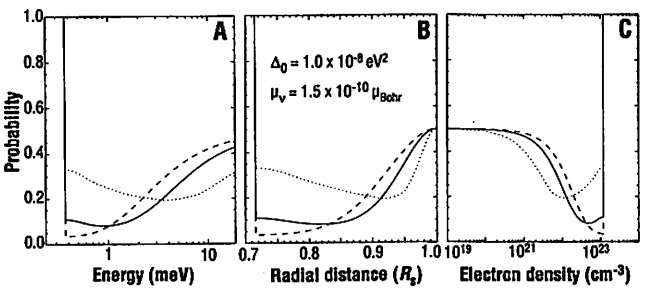

Fig. 5. (**A**) Model calculation of the probability of electron neutrino survival as a function of energy from the ^{71}Ga threshold (0.2332 MeV) to the cutoff energy for *hep* neutrinos (18.77 MeV) (*8*). The electron density is constructed from models in (*8*) and (*36*). The (toroidal) magnetic field is taken to vary as $B \sim n_e^{1/6}$ (n_e, number density of electrons) throughout the convective zone and is normalized to 2.5 kG at 0.91 times the radius of the sun (*24*); B_0 is set to 1.0 kG and the magnetic field is set to zero everywhere inside the convective zone [assuming the solar dynamo is confined there (*30*)]. The solid line shows the survival probability for "average" field conditions. The dashed line corresponds to a field strength that is 30% higher than average, and the dotted line, to a strength 30% lower than average for the neutrino parameters shown. (**B**) and (**C**) show the same probability as a function of the radial location of the spin-flip resonance and the corresponding resonant density, respectively.

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14. Data from runs 18 through 22, 24, 27 through 33, 35 through 92, and 94 through 99 are from tables 10.3 and 10.5 of (*8*). The data from nominal run 90 were used (showing an ^{37}Ar production rate of 0.0), and run 93 was not included. The ^{37}Ar production rates

for runs 100 through 103 were scaled from figure 1 of (7). The ^{37}Ar rates for runs 104 through 114 were scaled from figure 1 of (35). Data for runs 115, 116, 118 through 122, and 124 through 126 were taken from figure 6 of (1). The average solar neutrino capture rate over all 91 runs used differs slightly from the best average for all the available Homestake data (1); including Homestake data from before the IMP 8 launch gives a different average. In the joint Homestake–IMP 8 data, the average neutrino capture rate is 0.527 captures per day with a standard deviation of 0.315 captures per day.

15. Corresponding values for the solar wind flux are 3.91×10^8 and 0.67×10^8 cm^{-2} s^{-1} for the 91 points used in the two sets. There is no significant skew to either data set.
16. Values from the beginning of 1973 through the end of March 1991 were obtained originally from the Solar Environment Laboratory (National Atmospheric and Oceanic Administration) in Boulder, CO, and were updated through the end of September 1994 with daily sunspot numbers obtained from the National Geophysical Data Center by means of the World Wide Web.
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37. This work has taken place over a number of years. Analysis of the relevant solar wind data has been supported under a variety of contracts and grants from the National Aeronautics and Space Administration, including NAGW-1550 through task SC-A-276029 from MIT to Visidyne, task SC-A-383689 from MIT to the Johns Hopkins University Applied Physics Laboratory, under Task I of Navy Contract N00039-91-C-001. I especially acknowledge both the original principal investigator (PI) on the IMP 8 solar wind experiment, H. S. Bridge, the current PI, A. J. Lazarus, and K. I. Paularena (all of MIT) for access to, provision of, and discussions about the IMP 8 plasma data. J. McKinnon provided an initial set of daily sunspot numbers in 1991. I also thank J. Wakefield for calling my attention to the recent review of R. S. Raghavan. D. Rust and C. Keller referred me to the convection zone model of Spruit. I also thank E. Roelof for discussions and comments on the text, D. Rust and M. Dubin for their comments and suggestions, and P. R. Gazis for discussions and access to the PVO plasma data through the Ames Research Center Pioneer Project. Professor Bridge passed away 30 August 1995; this work would not have been possible without him.

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AFM Fabrication of Sub-10-Nanometer Metal-Oxide Devices with in Situ Control of Electrical Properties

E. S. Snow and P. M. Campbell

Metal wires and metal-oxide-metal junctions were fabricated by anodic oxidation with the conducting tip of an atomic force microscope (AFM). The width of the wires and resistance of the junctions were controlled by real-time, in situ measurement of the device resistance during fabrication. Because the properties of nanometer-scale devices are very sensitive to size variations, such measurements provide a more accurate method of controlling device properties than by controlling geometry alone. In this way, structures with critical dimensions of less than 10 nanometers were fabricated with precisely tailored electrical properties.

The electrical characteristics of device structures with feature sizes of 10 nm or less are extremely sensitive to variations in size. Because of this sensitivity to local geometry, reproducible device characteristics require near-atomic control of the fabrication. Although a few lithographic techniques can produce features in the 10-nm size regime (*1*), it is unlikely that one can achieve reproducible device properties in such small structures through control of device geometry alone. However, the use of a high-resolution processing technique in conjunction with real-time in situ measurement of device properties during critical fabrication steps as feedback for process control should allow the fabrication of devices with features smaller than 10 nm with precisely tailored electrical properties.

To implement such an approach, we need a fabrication process that does not interfere with the measurement of device properties during fabrication. One such process is atomic force microscope (AFM)–based anodic oxidation, a relatively new technique that has proven useful for lithography and device fabrication in the sub-100-nm size regime (*2*, *3*). An electrically biased AFM tip in contact with a surface is used under ambient humidity to produce a local surface oxide. Because the oxidation process produces no measurable current flow between the tip and sample, and because the technique can produce small feature sizes with no proximity effects (*2*), this process is well suited for the tailoring of small structures controlled by in situ electrical measurements.

In this report, we describe the use of in situ electrical measurements to control the fabrication of metal-oxide device structures with feature sizes ~10 nm. We used AFM anodic oxidation of thin Ti films to fabricate fine metal wires and Ti-TiO_x-Ti lateral junctions. Both the wire width and the junction resistance are controlled by in situ real-time measurement of the device resistance. In this way, metal wires with widths of 5 to 10 nm were achieved with predetermined resistance values. Such structures demonstrate the potential of in situ electrical measurements to produce devices of size ≤10 nm with precisely controlled electrical properties.

The scanning tunneling microscope (STM) under appropriate bias conditions can be used to selectively oxidize nanometer-sized regions of a H-passivated Si surface (*4*). A similar technique can be used to oxidize the surface of deposited Ti films (*5*); this oxidation process can completely penetrate suitably thin Ti films to produce lateral metal-oxide-metal device structures with variable junction resistance (*6*). Recently, such junctions were used to fabricate a single-electron tunneling device that was operational at room temperature (*7*). Because these and similar structures require feature sizes of order 10 nm, it is necessary to develop fabrication schemes such as de-

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scribed above if reproducible device properties are to be achieved.

To fabricate the wire and lateral oxide junction devices, we first used optical lithography and liftoff to pattern 7-nm-thick evaporated Ti films on a 60-nm-thick thermally grown SiO_2 layer on a Si wafer (8). Each metal pattern consisted of a wire (typically 2 to 4 μm in width) connected to large contact pads. After liftoff, the devices were cleaned in chemical solvents, mounted in a chip carrier, and wire-bonded. The bonded devices were then mounted in an AFM (Park Scientific Instruments Universal Ambient AFM). Electrical leads were attached to the chip carrier so that electrical measurements could be made on the devices during AFM operation.

For the oxidation step, we used Si Ultralevers (Park Scientific Instruments). The anodic oxidation proceeds through an electrochemical reaction that is driven by a negative electrical bias applied to the tip in the presence of ambient humidity (9). The devices shown here were fabricated in ~40% ambient humidity. The oxidation process does not produce any measurable current flow between the tip and sample and does not perturb the real-time measurement of the electrical characteristics of the device during oxidation. The electrical resistance of the device was used as an automated feedback control over the tip voltage to control both the width of fine metal wires and the resistance of lateral metal-oxide-metal junctions.

With the Si tips, we could oxidize the entire thickness of the Ti film (7 nm) with a tip-sample bias >10 V (the tip is biased negative with respect to the sample). The high resistance of a single metal-oxide-metal junction fabricated in this voltage range ($>10^{12}$ ohms) is evidence for complete oxidation. Smaller biases yield incomplete oxidation and lower junction resistances.

Such exposures to high voltage (>10 V) were used to define fine metal wires from the larger starting structures. First, the tip was biased at −12 V and scanned (scan speed s = 1 μm/s) over a rectangle that covered about half the width of the starting wire. This step restricted the current flow to half the width of the wire along a small section of its length. The tip was then positioned on the unoxidized side of the wire and scanned toward the oxidized region while the device resistance was monitored. During this step, the electrical resistance of the structure increased as the current was slowly constricted by the oxidation (Fig. 1A, inset). At a predetermined increase in resistance (ΔR), the tip voltage was automatically and suddenly dropped to zero, thus establishing a wire of known resistance whose width w was related to ΔR by $\Delta R = (\rho L/T)(1/w - 1/w_0)$, where w_0 was the initial width of the wire, L was the length of the wire, and ρ was the resistivity of the Ti film of thickness T.

Typical data from a measurement of ΔR versus time recorded during the oxidation process [the time is linearly proportional to the wire width, that is, $w(t) = w_0 - vt$, where v is the velocity of the tip perpendicular to the scan direction] are well fit (Fig. 1A) by the simple relation between ΔR and $w(t)$. This particular run was programmed to produce a 15-nm-wide by 500-nm-long wire. Because the oxidation produces a 2- to 3-nm swelling of the film, the Ti oxide can be imaged with the AFM (Fig. 1B). This image shows a narrow opening through the oxide, which has a width that is in approximate agreement with the value predicted by the resistance measurement (*10*). To test the path of the current, we oxidized a small section of the constriction at −12 V, which changed the resistance of the structure from ~10^5 to $>10^{12}$ ohms. Thus, there are no parasitic leakage paths, and all of the current flows through the channel defined by the oxidation.

Both the starting Ti film and the fabricated wires exhibit linear current-voltage (*I-V*) characteristics in the temperature range from 4.2 to 300 K. The only change in the electrical properties caused by the oxidation is an expected increase in resistance resulting from the constriction of the current flow to the narrow channel defined by the oxidized regions.

In addition to the narrow wires described above, it is also possible to fabricate lateral metal-oxide-metal junctions with controlled resistance. We monitored the device resistance while slowly increasing the voltage on the tip during repeated scans across the width of the wire. Above a

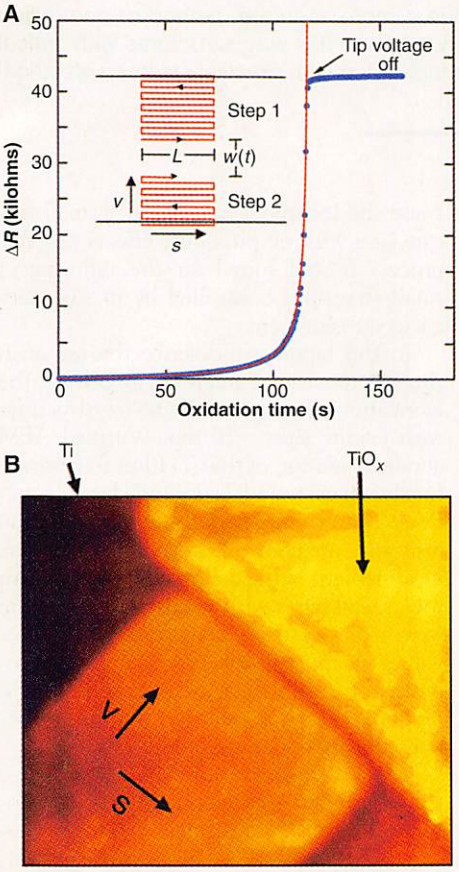

Fig. 1. (**A**) The measured change in device resistance recorded during fabrication of a constriction (blue dots) and a fit to the data (red line) assuming that the width of the constriction is a linearly decreasing function of time. (**Inset**) Diagram of the fabrication process. The wire has a width $w(t)$ as a function of time and a length L, s is the scan speed, and v is the tip velocity perpendicular to the scan direction. (**B**) An 850 nm by 850 nm AFM image of the finished structure. The observed pattern results from the swelling of the oxidized portions of the Ti film. The vertical color scale ranges 10 nm from black to white.

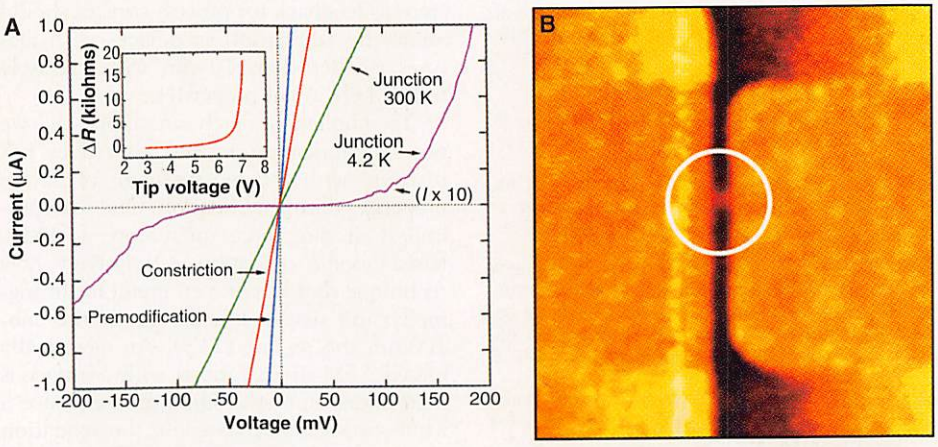

Fig. 2. (**A**) The *I-V* characteristics of an unpatterned film, a film with a 30-nm constriction, and a 30-nm-wide constriction with a single junction measured at 300 and 4.2 K. (**Inset**) The measured change in device resistance recorded during the fabrication of a junction. (**B**) A 900 nm by 900 nm AFM image of the constriction and oxide junction (indicated by the circle). The vertical color scale ranges 4 nm from black to white.

threshold voltage, the oxidation began to increase the wire resistance. When the desired junction resistance was achieved, the tip voltage was dropped to zero (Fig. 2A, inset). The value of the room-temperature junction resistance can be varied continuously, although for high-resistance junctions, the resistance changes rapidly, and it is thus more difficult to achieve a precise predetermined value.

For a single-junction device (Fig. 2B) with moderate resistance ($\sim 10^5$ ohms), the *I-V* curves at 300 K (Fig. 2A) were linear, but they became nonlinear for larger resistance values. At 4.2 K, the *I-V* characteristics exhibited a suppression of the conductivity around zero bias. We found that this low-temperature conductivity suppression is a general property of these lateral junctions. We also observed a correlation between the magnitude of the 300 K junction resistance and both the 4.2 K zero-bias resistance and the voltage range over which the suppression occurs. This suppression indicates a transition from the metallic-like behavior of the unoxidized wire to tunneling through the insulating oxide junction. The low-temperature, low-bias conductivity suppression was observed for all of the samples with lateral oxide barriers.

The fine wires and lateral tunnel junctions form the building blocks from which more complex electronic device structures can be constructed. An example is the single-electron tunneling device fabricated by Matsumoto (*7*), which consisted of a pair of STM-fabricated oxide junctions crossing a 30-nm-wide Ti wire. The ability to fabricate such structures with controlled electrical properties should facilitate the successful application of such devices.

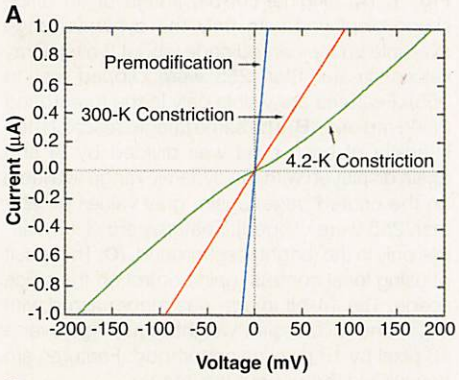

Fig. 3. (**A**) The *I-V* characteristics of an unpatterned film and a film with a constriction of <10 nm measured at 300 and 4.2 K. (**B**) An AFM image of the 120-nm wire and the narrow constriction. The vertical tick marks are spaced by 7.5 nm, and the horizontal tick marks are spaced by 100 nm.

To explore the minimum feature size that can be achieved by this technique, we fabricated a series of successively narrower wires and compared their *I-V* characteristics. We used as our signature of a good wire an *I-V* characteristic at 4.2 K that exhibits little or no zero-bias conductivity suppression, because such a suppression indicates that some portion of the wire has been inadvertently oxidized. To form the narrowest wires, we used a two-stage constriction process. The purpose of the first constriction (1 μm by 120 nm) was to provide a relatively narrow starting structure. Within this narrow region of the first constriction, we formed the second constriction by using only very fine tip translations. In this second constriction, the width was reduced by 0.5 nm with each successive scan, which resulted in fine control of the resistance of the constricted wire. The final result was a 200-nm-long wire of extremely narrow width (Fig. 3B). The narrowest wire we have fabricated by this technique was obtained with a resistance increase that corresponds to a final wire width of 3 nm. The *I-V* characteristics of this device (Fig. 3A) show only a slight suppression of the zero-bias conductivity at 4.2 K, which indicates that the 300 K resistance is not dominated by oxide junctions extending across the wire.

For such narrow wires, the resistance measurement may lead to an underestimate of the actual geometric width. One indication of this is that the in situ resistance data were no longer fit by a simple linearly decreasing wire width. This error could have resulted from a variety of sources, most important of which may be nonuniformities caused by the grain size of the metal film. Although the linearity of the *I-V* characteristics indicated no dominate oxide junctions, the finite grain size of the metal produces nonuniformities of similar size in the oxide (Fig. 3B). These nonuniformities lead to width fluctuations that may dominate the resistance and result in an underestimation of the average width of the wire. Further reductions in size will require more uniform metal films. Additional errors could arise because the intrinsic resistivity of Ti increases as the film is constricted to very narrow dimensions. Such a change in resistivity in long, narrow wires has been noted by researchers who formed small metal wires by pressing an STM tip into a metal substrate and slowly retracting it (*11*).

An AFM image from after oxidation (Fig. 3B) indicates that the swelling from the oxidation just bridges the channel for this particular structure. This contrasts with the device in Fig. 1, which has a 15-nm-wide effective electrical width and an ~10-nm-wide gap in the surface swelling. Although this gap cannot be seen in the images of the narrowest wires, the lack of significant zero-bias conductivity suppression indicates the presence of an unoxidized Ti wire buried beneath the surface oxide. On the basis of the AFM images and the resistance data, we estimate this wire to be between 5 and 10 nm wide. The electrical properties of these devices have been precisely and controllably determined, although their geometrical dimensions are not measurable to this precision.

We have combined proximal probe-fabrication techniques with in situ electrical measurements to produce 5- to 10-nm-wide metal wires and lateral metal-oxide-metal junctions with controlled electrical properties. The ability to fabricate structures in this size regime with such finely tailored electrical properties may allow the realization of useful devices whose operating characteristics are critically dependent on device geometry but for which control of geometry alone would be difficult if not impossible to achieve by conventional nanofabrication methods.

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10. The exact dimensions of the wire are obscured by several effects. First, the AFM image indicates a rounding of ~20 nm in radius at the edges of the oxide; this rounding is the result of both an actual rounding of the oxide edge and tip convolution effects. Second, the profile of the oxide below the surface is unknown. These effects lead to an uncertainty in the width of the electrically active region. In this size regime, the electrical measurements appear to be the more accurate estimate of the width. For constrictions down to 10 to 20 nm, the resistance data are well fit assuming the wire width decreases linearly with time. Consequently, we take as our estimate of the width the value established by the resistance.
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Image Enhancement with Polymer Grid Triode Arrays

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An array of polymer grid triodes connected by a common grid functions as a "plastic retina," providing local contrast gain control for image enhancement. This simple device, made from layers of conducting polymers, functions as an active resistive network that performs center-surround filtering. The polymer grid triode array with common grid is a continuous analog of the discrete approach of Mead, with a variety of fabrication advantages and significant savings in area within the unit cell of each pixel.

When a person views a brightly lighted external scene through a window from inside a poorly lighted room, the individual has no difficulty seeing simultaneously the details of both the internal scene and the external scene. This is done by local contrast control; the visual system locally adjusts the gain using lateral inhibition.

Consider the office scene in Fig. 1. When the original (14-bit) image is displayed with only the dynamic range available on the printed page (about 8 bits) (Fig. 1A), gray regions greater than 255 are clipped (set to 255), simulating saturation in the region of highest brightness. When the image is rescaled (the intensity of each pixel was divided by 8) and displayed over the same dynamic range (Fig. 1B), it is analogous to the image shown on a video display with reduced gain; the features are visible only in the background (bright) regions. In both cases, a great deal of information contained in the original (14-bit) image is lost: in Fig. 1A, the viewer cannot see any detail in the bright regions of the image, and in Fig. 1B, the viewer cannot see any detail in the darker regions of the image.

Local contrast control involves a combination of logarithmic compression and lateral inhibition, the latter provided by a horizontal resistive network (a neural network) (*1*, *2*). After logarithmic compression, the output (V_i) from a given pixel i is proportional to the log of the intensity (I_i) of that pixel

$$V_i = V_0 \log I_i \qquad (1)$$

where V_0 is the constant of proportionality. Lateral inhibition is implemented by subtracting from V_i the average of the surrounding values; thus, the renormalized image is defined by

$$v_i = V_0 \log I_i - \langle V_0 \log I_i \rangle = V_0 \log I_i - \frac{V_0}{N}\sum_i \log I \qquad (2)$$

where the brackets denote an average value and the sum is over neighboring pixels within the averaging range (the center-surround or blurring range). Equation 2 is equivalent to

$$v_i = V_0 \log \left[\frac{I_i}{(\prod I_i)^{1/N}} \right] \qquad (3)$$

The denominator is the geometric mean. Equation 3 is the mathematical expression of Mead's local contrast enhancement algorithm (*1*); it can be generalized to allow for a weighted average by replacing $1/N$ by w_i, where w_i are the weights.

If the original image of the office scene is processed with the Mead algorithm (Fig. 1C), one can see details in the entire image even within the limited range available on the printed page. The simulations in Fig. 1 demonstrate the power of local contrast enhancement.

In this report, we show that the local contrast enhancement algorithm (Eqs. 2 and 3) can be implemented with a simple device made from layers of conducting polymers. The device consists of an array of polymer grid triodes (PGTs) (*3*) connected through a common grid, which serves as a resistive network.

In a layered thin-film PGT (Fig. 2A), the top layer (**5**) is the anode and the bottom layer (**1**) is the cathode. The third electrode (**3**), analogous to the grid in a vacuum tube triode, is an open network of polyaniline (PANI) protonated to the highly conducting form with camphor sulfonic acid (CSA) (*3*). Semiconducting polymer forms layers **2** and **4**, between the anode and the polymer grid and between the polymer grid and the cathode, and fills the void spaces within the porous PANI-CSA network (**3′**).

An array of PGTs with a common grid (Fig. 2B) can perform local contrast enhancement like that simulated in Fig. 1. Because the thin films that constitute the PGT array can be processed from solution, they can be layered directly on an array of photodetectors. Each node of the PGT array corresponds to one pixel of the image. The array of PGTs with a common grid performs three important functions: (i) The common grid functions as a resistive network that computes the blurring (averaging) in Eq. 2. (ii) The output current at one node of the PGT array is approximately the difference between the input anode-to-cathode voltage and the local grid voltage. Because the local grid voltage is the local average [see (i)], the PGT array acts as a center-surround filter (*1*, *2*) that computes the difference in Eq. 2. (iii) The PGT array with common grid provides the high input resistance needed for open circuit detector operation (*4*), which results in the logarithmic compression in Eq. 2.

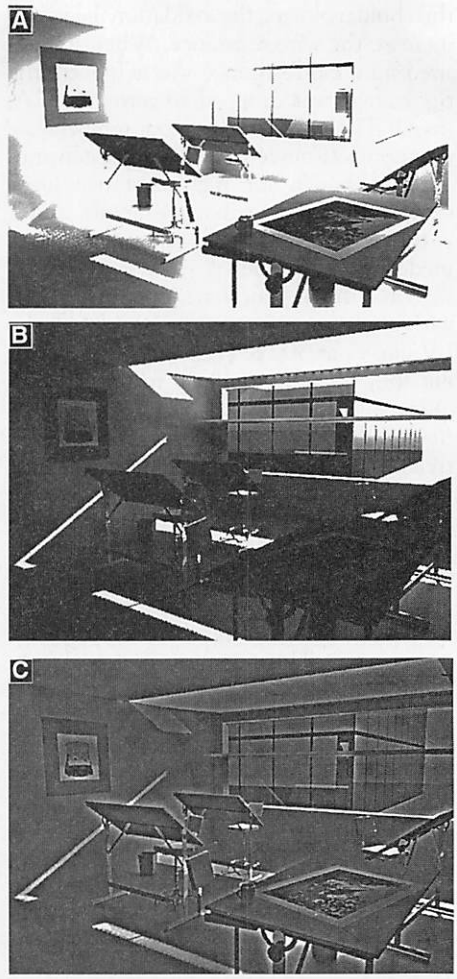

Fig. 1. (**A**) Original (14-bit) image of an office scene displayed with only the dynamic range available on the printed page (about 8 bits). Gray values greater than 255 were clipped (set to 255). Features are visible only in the foreground (dark) regions. (**B**) The same image, rescaled (the intensity of each pixel was divided by 8) and again displayed with the dynamic range available on the printed page (again, gray values greater than 255 were clipped). Features are clearly visible only in the (bright) background. (**C**) The result of using local contrast gain control on the office scene. The 14-bit image was renormalized with Eq. 3 and a Gaussian-weighted average over a 13 pixel by 13 pixel neighborhood. Features are now visible throughout the image.

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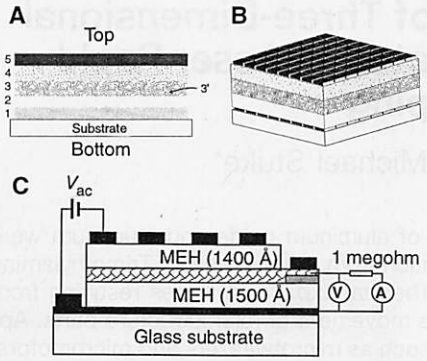

Fig. 2. (**A**) Structure of the PGT. Layers **1** and **5** are the cathode and anode (pixel) arrays, respectively. The other layers are continuous films common to all the PGTs within the array: **2** and **4** are semiconducting layers, poly(2-methoxy-5-(2′-ethyl-hexyloxy)-1,4-phenylene vinylene) (MEH-PPV), and **3** is the common grid network filled with semiconductor (**3′**). (**B**) Schematic diagram of an array of PGTs with a common grid. Thickness (between anode and cathode) is about 0.3 μm. Each anode-cathode pad is, for example, 50 μm on a side. (**C**) Four PGTs in an array with a common grid.

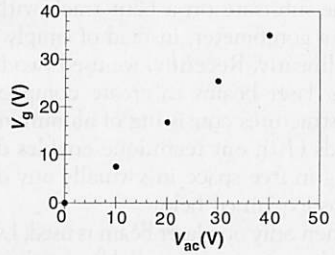

Fig. 3. Grid voltage V_g is proportional to input voltage applied at neighboring positions, that is, the grid voltage provides a local average; the voltage V_{ac} is applied between the anode and cathode on the left of the array, and V_g is measured near the rightmost triode of the array (Fig. 2C).

Thus, local contrast enhancement, as described by Eqs. 2 and 3, can be directly implemented with the PGT array in Fig. 2B. The effective computation rates involved are impressive: The equivalent calculation implemented on a serial computer would require about 40 million multiplications per second (256 × 256 pixels, 30 frames per second, 13 × 13 blurring with separable convolution).

We have fabricated PGT arrays with four triodes on a single substrate, all with a common grid (Fig. 2C). The fabrication process for the triode array is similar to that of a single PGT (*3*); the principal difference is that there are separate contact pads for each device in the array. For the arrays fabricated in this study, the sheet resistance of the common grid was about 20 kilohms per square.

The voltage of the common grid (V_g) with respect to the anode was measured

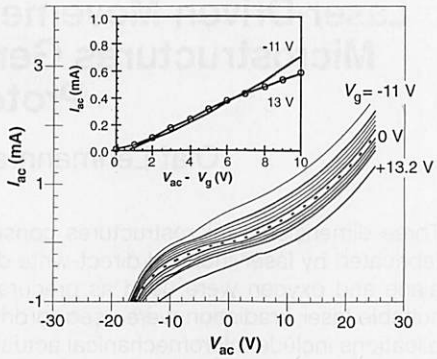

Fig. 4. The I_{ac} versus V_{ac} curves for different grid voltages (V_g). (**Inset**) The two limiting data sets [V_g = −11 V (solid line) and V_g = +13.2 V (line with circles)] as a function of $V_{ac} - V_g$.

while a voltage (V_{ac}) was applied between the anode and the cathode of the left triode (Fig. 2C). The voltage of the common grid was measured near the neighboring triode on the far right; V_g at this neighboring position responds in proportion to V_{ac} (Fig. 3) such that $V_g = \beta\langle V_{ac}\rangle$ (where β is a constant of proportionality), that is, the grid voltage responds to the local input and provides a local average.

The final step required to demonstrate the validity of the PGT implementation of local contrast enhancement is to show that it computes the center-surround difference in Eq. 2. The equivalent circuit of the PGT (*3*) is two coupled diodes connected back-to-back, like a bipolar transistor (*4*). This is achieved by using semiconducting polymer in layers **2**, **3′**, and **4**. For the prototype array sketched in Fig. 2C, layer **2** was fabricated with a material of sufficient conductivity to make an ohmic contact to the grid, so that the equivalent circuit is simplified to a diode in series with a resistor. In the initial experiments, polyvinylcarbazole (PVK) was used for resistor layer **2**. In forward bias

$$I = I_0 e^{\gamma(V_{ac} - V_g)} + \frac{(V_{ac} - V_g)}{R_s} + \frac{V_g}{R_i} \qquad (4)$$

where γ is a constant (*3*), R_i is the internal series resistance of the diode (from the bulk resistivity of the semiconducting material used in **4**), and R_s is the series resistance resulting from the PVK layer (**2**). Because the semiconducting layer is fabricated from a high-resistivity, pure semiconducting polymer such as poly(phenylene vinylene) (PPV) or one of its soluble derivatives, $R_s \ll R_i$. Thus, the output of the PGT is a function of $V_{ac} - V_g$ only; that is,

$$I = F(V_{ac} - V_g) \qquad (5)$$

This is demonstrated by I versus V_{ac} data for different V_g (Fig. 4). As expected, the curves are sensitive to V_g: for example, at $V_{ac} \approx 5$ V, the current can be suppressed from 1 mA to zero by changing V_g. In the inset to Fig. 4, we replotted the curves from the two limiting data sets ($V_g = -11$ V and $V_g = +13.2$ V) as a function of $V_{ac} - V_g$. Because the forward-bias data collapse onto a single curve, Eq. 5 is indeed valid. Consider then the array of PGTs, with common grid, sketched in Fig. 2B, and assume, for simplicity, that the common grid is grounded at infinity. Because of the common grid

$$V_g = \beta\langle V_{ac}\rangle \qquad (6)$$

where $\langle V_{ac}\rangle$ denotes the average over neighboring pixels with a distance determined by the sheet resistance of the grid and the conductance to ground of the resistive layer (in this case, PVK). The characteristic length over which the average is taken (*1*) is given by

$$L = 1/(\rho\sigma)^{1/2} \qquad (7)$$

where ρ is the sheet resistance of the PANI network grid and σ is the conductance per unit area to ground through the resistive PVK layer.

We conclude that the current output from each pixel of the array is given by

$$I_i = F(V_{ac}^{(i)} - \beta\langle V_{ac}\rangle)$$
$$\approx (V_{ac}^{(i)} - \beta\langle V_{ac}\rangle)(\partial F/\partial V) \qquad (8)$$

Equation 8 is equivalent to Eq. 3 provided that the output of each detector on the focal plane array $V_A^{(i)}$, which serves as input to an individual pixel i, is proportional to the logarithm of the intensity: $V_A^{(i)} \propto \log I_L^{(i)}$, where $I_L^{(i)}$ is the intensity of the light incident on the ith pixel. Because $V_{out} \propto \log I_L$ for photovoltaic detectors under open circuit conditions, the logarithmic compression of Eq. 1 is straightforward (*4*).

The PGT-array image processor differs fundamentally from those built with discrete silicon field-effect transistors (*1*, *2*). The PGT array makes use of the spreading resistance of the PANI control-grid network to provide the interconnection of a given node to its neighbors; the conductivity of the PANI network enables center-surround filtering as a result of lateral charge redistribution initiated by contrast differences. Charge redistribution through a continuous layer of material provides a natural means for averaging (blurring).

By controlling the concentration of PANI in the network, one can control the resistivity over many orders of magnitude (*5*). Similarly, by varying the thickness and the resistivity of layer **2**, by back biasing the grid-to-ground diode during operation or by making **2** a bilayer that functions as a diode with the use of conducting polymers (*6*), one can vary the conductance of layer **2** over a wide range. The latter is particularly

interesting because it allows in situ dynamic control of the spatial decay length. Thus, one can vary both ρ and σ (Eq. 7) so as to achieve values for L (either statically or dynamically) ranging from a few micrometers to 1 cm.

We have shown that this simple device, made from layers of conducting polymers, provides both logarithmic compression and lateral inhibition of response, as required for local contrast control. Nevertheless, the plastic retina is at an early stage of development. The utility of the PGT array for image enhancement will depend on a number of factors—including, for example, sensitivity, noise, dynamic range, and matching from one pixel to the next—that must be tested on an engineering prototype.

For a full plastic retina, the PGT image enhancement array would be fabricated directly onto the output side (back) of a photodetector array (for example, an infrared detector array) with each detector output pad as the anode or cathode of the PGT at that node. The semiconductor layers would be cast sequentially from solution and applied onto the detector array much like an antireflection coating. The final contrast-enhanced output would be connected to a demultiplexer by "bump bonding"; that is, by cold-welding indium bumps arrayed reciprocally on the PGT array output and on the demultiplexer input.

Alternatively, the PGT array could be used to process the image after analog-to-digital conversion and integrated directly into a display (such as a liquid-crystal display). In this case, the PGT array would be fabricated directly on, as an integral part of, the display: for example, between the control circuits and the liquid-crystal layer. The data would be logarithmically compressed digitally and input into the PGT array to process the image; the output from the pixels of the array of PGTs would serve as the input to the pixels of the display.

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Laser-Driven Movement of Three-Dimensional Microstructures Generated by Laser Rapid Prototyping

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Three-dimensional microstructures consisting of aluminum oxide and aluminum were fabricated by laser-induced direct-write deposition from the gas phase. Trimethylamine alane and oxygen were used as precursors. Thermal expansion forces resulting from suitable laser irradiation were used to drive the movement of microstructure parts. Applications include micromechanical actuators, such as microtweezers and micromotors. The one-step nature of the laser direct-write process allows rapid prototyping of such devices.

Lithography and etching techniques that were originally developed for microelectronics applications can also be used to fabricate sensors, actuators, or other micromechanical devices on silicon. However, these techniques require photo masks to be made before the microstructures can be produced, and the production itself consists of many complicated and time-consuming steps—for example, because vertical structure variations can be performed only indirectly. Structural redevelopment often requires redesign of the masks. As a result, such techniques are economical mainly for mass production of devices that do not need any further development. Other mask-based techniques, such as the x-ray deep etching technique LIGA (Lithographie, Galvanoformung, Abformung), also suffer from this drawback.

As a result, methods for the single-step generation of three-dimensional (3D) microstructures have recently received considerable attention. The idea is to design a structure with the use of computer-aided technologies and to transfer the data directly to a machine, which then automatically generates a solid structure. This so-called rapid prototyping enables very fast evolution of structures, particularly when laser direct writing is used as a processing tool. Techniques based on photopolymerization or powder sintering have already been used in rapid prototyping of commercial products, but they offer only submillimeter resolution. Another possibility is fast layer-by-layer laser etching of silicon in chlorine, which can be performed with a resolution of 1 μm (*1*).

Laser-assisted chemical vapor deposition (LCVD) has been shown to be suitable for rapid prototyping with micrometer resolution (*2*). The method enables fabrication of thin rods and fibers by pulling the substrate away from the stationary laser focus at a speed equal to the linear growth speed of the material, keeping the laser focus on the rod tip (*3*). LCVD was first demonstrated for carbon (*4*) and silicon (*5*) rods, but it has also been applied to the production of long carbon (*6*), silicon (*7*), and boron (*8*) fibers. More complex structures such as microsprings (*9*) have been obtained by moving the substrate on a bent track with the use of a goniometer, instead of simply pulling it linearly. Recently, we used two intersecting laser beams to create complex 3D microstructures consisting of aluminum oxide rods (*10*); our technique enables direct writing in free space in virtually any direction, as explained below.

When only one laser beam is used, LCVD can only create rods parallel or nearly parallel to the incident beam. To obtain rods in any direction, it was necessary to modify the technique. In weakly absorbing materials such as alumina, the absorption length of the laser light is large; the resulting uniform temperature distribution in the illuminated region leads to nondirectional material depo-

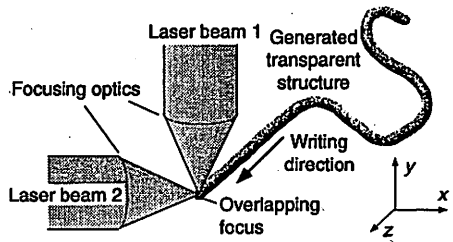

Fig. 1. Three-dimensional direct-write principle. The nearly transparent alumina rod absorbs the laser light only weakly, and thus most of the energy is absorbed in the bulk rather than at the surface. The laser beams are attenuated so that almost no growth is observable for either beam alone. The temperature rise causes nondirectional alumina deposition from the gas phase only in the area of overlapping focus, where the temperature threshold is exceeded. The writing direction is defined by slow (~10 μm s^{-1}) movement of the laser focus.

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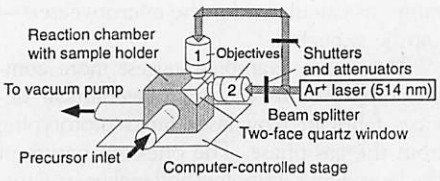

Fig. 2. Experimental setup. The reaction chamber has two tubular connections for precursor supply and pump-out, respectively. It is fixed on a computerized scanning table that can also be controlled manually by a joystick. The structure and the substrate surface are mounted inside the chamber and can be inspected with an optical microscope through a two-faced quartz window. A second objective lens allows observation from the side at a 90° angle. The laser beam, emitted from an argon ion laser (Spectra Physics 2030) working at 488 or 514 nm, is attenuated, split into two separate beams, and, after passing two computer-controlled mechanical shutters, focused through the two microscope objective lenses (magnification ×8, numerical aperture 0.2). The focal spots (diameter, ~3 μm) are adjusted to overlap, which defines the reaction zone. The shutters enable selection of one or both laser beams.

sition from the gas phase around the laser spot. If an additional focused laser beam perpendicular to the first one is adjusted so that the two spots overlap, and if the laser power is low enough to prevent considerable growth for only one beam, the deposition region is well defined by the intersection of the laser spots (Fig. 1). At this intersection, the absorbed energy is sufficient for a temperature rise that causes deposition from the gas phase. If the sample is moved slowly in any direction with a speed comparable to the growth rate of alumina, a structure with a complex shape can be directly written into free space.

Complex structures previously produced in this way are static, because every part is fixed directly or indirectly to the substrate. Here, we used this LCVD-based technique to build complex movable structures, such as a simple linear "micromotor," that are driven by thermal expansion forces caused by selective laser beam heating of structure parts. Devices of this kind can serve as micromechanical actuators.

The 3D structures described below were built on suitable substrates that were mounted inside a small reaction chamber on a turnable holder (Fig. 2). The effective laser power for the experiments was varied between 0.2 and 20 mW. The aluminum precursor trimethylamine alane [TMAA, $AlH_3{\cdot}N(CH_3)_3$] was pumped slowly through the chamber, which was maintained at a total pressure of ~1.6 mbar. For the production of aluminum oxide (Al_2O_3) rods, oxygen was used as an additional gas with a partial pressure of ~0.2 to 0.6 mbar. The dependence of structure growth on several parameters, such as oxygen partial pressure, has been described elsewhere (*10*).

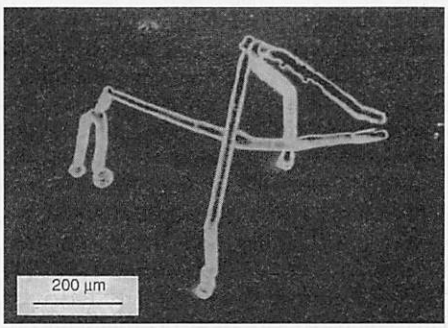

Fig. 3. Microtweezers fabricated by laser rapid prototyping. The tips of the tweezers (right) are moved together by the lifting of the lower lever, which is induced by laser heating and linear expansion of the right-hand rod in the pair of vertical rods (left). The long lever shifts the maximum travel to ~20 μm. The whole structure was fabricated within several minutes.

Local laser heating of structure parts in a nonreactive atmosphere can produce thermal expansion effects that lead to slight bending of the parts. Because the linear expansion coefficient of aluminum is considerably higher than that of Al_2O_3, rods that were designed to expand and apply force on other structure parts were coated with aluminum. Because the thermal conductivity of aluminum is much higher than that of Al_2O_3, the coating provides a more uniform temperature distribution that prevents local heat peaks, which could damage the structure.

Lever constructions can be used to increase the distance of travel, and we built a set of microtweezers (Fig. 3) on the basis of this principle. With the use of a suitable lever, we were able to multiply the linear expansion of the laser-heated rod (~1 μm) by a factor of 20. The force the microtweezers can apply is currently limited by the flexibility of the lever that holds the lower tip. The applied force F can be calculated from

$$F = \frac{3\pi r^4 z E}{4L^3} \quad (1)$$

where r is the radius of the lever, E is Young's modulus of alumina (5.2×10^{11} N m^{-2}), and L is the length of the lever (*11*). For r = 7 μm and L = 400 μm, and assuming a maximum bending of z = 5 μm, the force F is on the order of 0.1 mN—that is, more than 1000 times the weight of the tweezers. The maximum force may be further limited by the stability of the connection between the lever holder and the substrate.

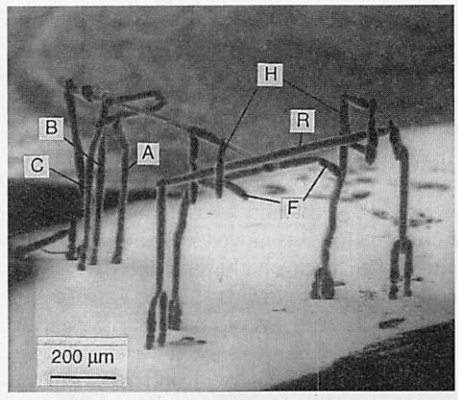

Fig. 4. Electron micrograph of the linear micromotor. The driving unit, consisting of three expandable rods (A, B, and C), is located at the back left. In the foreground, two square-ring holders (H), the horizontal movable rod (R), here still fixed to the substrate after fabrication and the fork (F) connected to the driving unit can be discerned.

The different thermal expansion coefficients of aluminum and alumina can also be used to cut rod connections to the substrate. When two parallel vertical aluminum-coated rods connected at the top are alternately laser-heated, the thermal strain leads to disconnection of the rod bases from the substrate. Freely movable structure parts can thus be manufactured by releasing them from the substrate after their shape has been defined by 3D laser direct writing.

Using the principles described above, we developed a simple linear micromotor with a maximum travel of ~100 μm (Fig. 4). The driving unit of the motor consists of three expandable rods that can be operated selectively. When the three rods are heated in a defined sequence, a closed movement of the horizontal rod R results (Fig. 5). The expansion of rods A and C bends the structure slightly to the left or to the right, respectively; hence, point P is moved to the left or to the right. When the laser beam is switched off, the shape of the structure re-

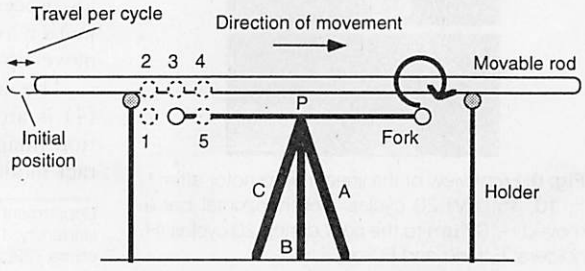

Fig. 5. Working scheme of the laser-driven linear micromotor (Fig. 4). A laser beam is scanned across the rods A, B, and C, causing a circular movement of the fork. During this cycle, the movable rod is lifted from the holders, moved to the right, and placed back on the holders.

turns to its initial state. Rod B is used for vertical movements; heating this rod results in a lift of point P. All travel distances are increased by lever constructions to maximum values of 20 μm at the lever end.

When a slightly defocused laser beam (diameter ~50 μm) is scanned from right to left over rods A, B, and C, the freely movable horizontal rod R is moved ~5 μm to the right and placed back on the holders. Each cycle consists of the following steps: (i) Heating of rod A moves the lever to the left. (ii) Heating of rods A and B moves the lever up; rod R is lifted from the holders H and rests on the lever fork F. (iii) Heating of only rod B results in the return of rod R to the middle horizontal position. (iv) Heating of rods B and C moves the lever further to the right. (v) Heating of only rod C lowers the lever; rod R is placed back on the holders H, but the position is shifted to the right relative to the initial state. (vi) Cessation of heating of rod C restores the horizontal beginning position of the lever. The motor is now ready for a new cycle. Repeating the cycle results in longer distances of travel (Fig. 6).

The working principle of the micromotor resembles the contraction of a muscle through the interaction of myosin and actin (*12*). The maximum force the motor can apply is limited in this simple model by the adhesion of the horizontal bar to the fork during the movement. This adhesion force, ~10 nN, is on the order of the weight of the bar. If this adhesion is increased (for example, by a rougher surface), much higher values of the force—in the micronewton range, as calculated for the microtweezers—can be expected.

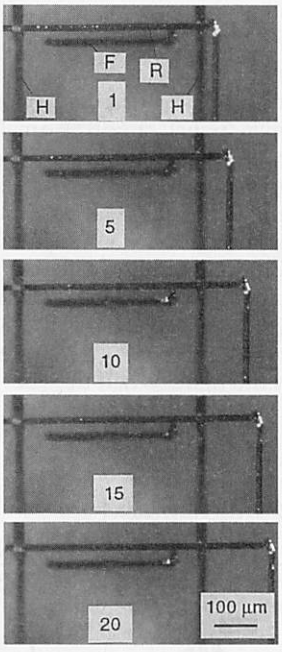

Fig. 6. Front view of the linear micromotor after 1, 5, 10, 15, and 20 cycles. The horizontal bar is moved ~100 μm to the right during 20 cycles (H, holders; F, fork; and R, rod).

The above examples suggest more complex applications for micromechanical devices fabricated by laser rapid prototyping from the gas phase. The one-step nature of the laser direct-write method makes structural evolution quick and easy, in contrast to mask-based processes. If focused laser light is used as the energy source, energy can easily be transferred to the moving structure; this would enable the creation of free "walking" devices with no mechanical connection to a battery or other energy source.

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Defect Motion on an InP(110) Surface Observed with Noncontact Atomic Force Microscopy

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With an atomic force microscope operating in the noncontact mode in an ultrahigh vacuum, atomic-resolution imaging of the cleaved semi-insulating InP(110) surface has been achieved. By this method, atomic scale point defects and their motion were observed at room temperature, without the field-induced effects associated with scanning tunneling microscopy.

The ability to resolve individual atoms over a wide area makes the scanning tunneling microscope (STM) an excellent tool for studying the migration of atoms or defects. However, in all real-time STM experiments of surface dynamics processes, there is a high probability that the motion is nonthermal and is instead affected by the electric field or tunneling current; that is, the migration of the atoms or defects is the result of an excitation by the electric field of the biased tip or an energy injection by the tunneling current. It is well known that manipulation of single atoms is possible with the STM (*1*). Furthermore, STM tip–induced motion of the atoms or defects can exceed the thermal motion at room temperature (*2*, *3*). To investigate surface dynamics processes related to thermally induced motion of atoms or defects, one must remove tip-induced nonthermal motion.

The atomic force microscope (AFM) (*4*) is an alternative tool for high-resolution imaging. When operating in noncontact mode, it is expected to be free of such nonthermal effects and therefore to be effective for observing thermally induced motion in real time, because its operation is based on weak interactions attributable to attractive forces, rather than the electric field utilized in the STM. So far, the lateral resolution of noncontact ultrahigh-vacuum (UHV) AFM has been insufficient because of technical difficulties in measuring the weak distance dependence of the attractive forces between tip and sample, which have the high signal-to-noise ratio of the force measurements. Recently, the adatoms of the Si(111) 7×7 reconstructed surface were resolved locally by AFM, but stable imaging was not achieved (*5*). Furthermore, the distances between the adatoms and the corrugation heights with corner holes on the Si(111) 7×7 reconstructed surface are relatively large compared with those of other semiconductor surfaces. Continuing technical improvements increasing the lateral resolution of noncontact UHV AFM are required.

In this report, we present noncontact UHV-AFM images with atomic resolution for the semi-insulating InP(110) surface. Atomic scale point defects have been ob-

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served at room temperature, and thermally induced motion of atoms or defects have been studied in real space.

We have used a very compact UHV AFM that allows in situ sample cleavage (6). The cantilever was scanned by a tube piezoelectric scanner, and its deflection was detected by an optical-fiber interferometer (7), which is one of the most sensitive displacement sensors. The frequency modulation (FM) detection method (8) was used to measure the force gradient acting on the tip. The tube piezoelectric scanner was also used to vibrate the cantilever at the mechanical resonant frequency. A positive feedback system with automatic gain control was used to maintain a constant vibration amplitude. The frequency shift of the cantilever was detected by a tunable analog FM demodulator.

As a force sensor, a silicon cantilever was used. When the attractive force gradient acting on the tip exceeds the spring constant of the cantilever, the cantilever jumps into the sample. Therefore, a stiff cantilever with a spring constant of $k = 34$ N/m and a mechanical resonant frequency of $\nu_0 = 151$ kHz was used to keep the cantilever from jumping and crushing the initially sharp tip. The sample was an Fe-doped semi-insulating InP(001) wafer (semi-insulating semiconductors cannot be studied by STM because of their lack of electrical conductivity). Sample cleavage and subsequent noncontact AFM measurements were performed at room temperature at pressures of less than 4×10^{-8} Pa. The images were obtained at constant force gradient; we kept the distance between the tip and the sample constant by maintaining a constant frequency shift $\Delta\nu$.

In an example of an actual image of a cleaved InP(110) surface by noncontact AFM (Fig. 1), the entire scan area is clearly resolved, including the rectangular lattice, which is slightly distorted as a result of thermal drift and creep of the piezoelectric micropositioners. In contact AFM imaging, the tip sticks to the sample at the beginning of the lateral scan (just after the lateral scan direction is reversed) because of static friction between tip and sample, and therefore, the observed atomic lattice periodicity is strongly distorted. In Fig. 1A, no such distortion occurs because noncontact imaging removes the friction effect between the tip and the sample surface.

The InP(110) surface is characterized by quasi–one-dimensional zigzag chains consisting of alternating In and P atoms (Fig. 1B) (2, 9). The electrons in the In dangling bonds partially transfer to the P, and the P atoms move out of the surface, resulting in a buckling. The lattice constants for the unit cell are 5.87 and 4.15 Å along the [001] and [1$\bar{1}$0] directions, respectively (2, 9). We have measured distances between the protrusions along the [001] and [1$\bar{1}$0] directions of 5.8 ± 0.6 and 4.3 ± 0.4 Å, respectively, in good agreement with the lengths for the unit cell. At present, we cannot clearly resolve the quasi–one-dimensional zigzag chains. The corrugation amplitudes were measured to be 0.19 ± 0.05 and 0.12 ± 0.05 Å along the [001] and [1$\bar{1}$0] directions, respectively. These amplitudes are about one-sixth of those obtained in contact-mode AFM (10). Note that the measured corrugation amplitudes depended on imaging parameters such as vibration amplitude A_0 and frequency shift $\Delta\nu$.

We have also been able to image atomic defects (Fig. 1A, feature a) reproducibly. Bright points on both sides of the atomic defects (features b and c) appear to be adsorbates. In Fig. 2, point defects are observed as atomic scale depressions (dark contrast) with respect to the background, suggesting that atoms have been removed from the surface. According to previous STM results (11) for the conducting InP(110) surface, these depressions may be "missing P defects" or "P vacancies"; we cannot verify the nature of the defects because the registry of the In and P sublattices is not resolved in the present AFM measurement. The defects may be caused by cleavage of the sample. The depressions observed in our measurements had approximate lateral dimensions of one unit cell in the [1$\bar{1}$0] direction, whereas vacancies observed with the STM broadened up to 4 nm as a result of the charge-induced band bending (2).

Occasionally, atomic scale point defects changed their relative position. In Fig. 2A, point defects d and e were separated by two atomic rows in the [001] direction. About 80 s later (Fig. 2B), defect e had split into two point defects, which were separated from defect d by three and four atomic rows, respectively, in the [001] direction; also, the distance between the point defects had decreased. Such position changes were studied for cases in which two or more defects were visible simultaneously; therefore, they do not reflect position changes of atoms on the tip apex because damage of the tip apex should affect all defects equally. The position change of the atomic scale point defects therefore originates from migration of the vacancies.

Our noncontact AFM observations show that vacancies can move not only within zigzag chains but also between zigzag chains. Experiments with a STM have

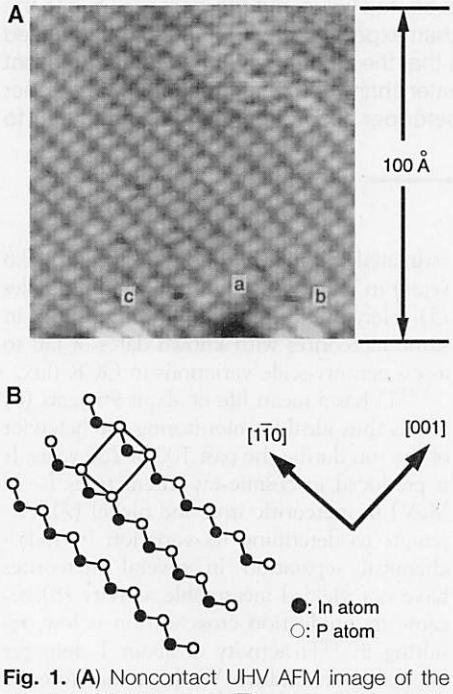

Fig. 1. (**A**) Noncontact UHV AFM image of the cleaved InP(110) surface. The scan area was 100 Å by 100 Å. Experimental conditions: spring constant of the cantilever $k = 34$ N/m, mechanical resonant frequency $\nu_0 = 151$ kHz, vibration amplitude $A_0 = 20$ nm, and frequency shift $\Delta\nu = -6$ Hz. Atomic defects (a) and adsorbates (b and c) are evident. (**B**) Surface structure of the InP(110) surface. The rectangle indicates a unit cell with lengths of 5.87 and 4.15 Å along the [001] and [1$\bar{1}$0] directions, respectively.

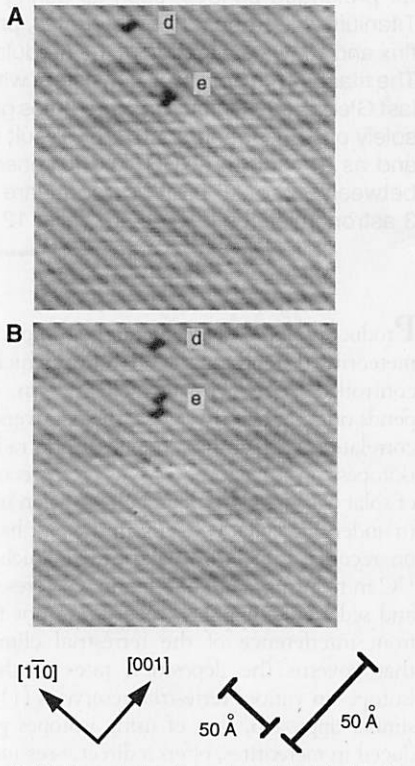

Fig. 2. Motion of atomic scale point defects. The fast scan direction was from left to right, and the slow scan direction was from bottom to top. (**A**) Point defects d and e are separated by two atomic rows in the [001] direction. (**B**) The same area imaged 80 s later. Defect e has split into two point defects, which are separated by three and four atomic rows, respectively, from defect d in the [001] direction. The distance between defects d and e has decreased.

shown that As vacancies on the GaAs(110) surface preferentially move between zigzag chains (*12*). This phenomenon can be understood in terms of simple bond-breaking and rebinding arguments (*12*). The observed migration of vacancies can be explained by the same arguments.

The motion of the defects could be thermally induced or the result of mechanical transfer of the atoms by the tip. In some cases, during the continuous observation of the same area, defects appeared and disappeared. The position changes were independent of the scanning direction, which indicates a thermal origin for the defect motion, but the present data is not sufficient to exclude other mechanisms. Quantitative comparison with STM imaging is necessary.

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Behavior of the Heliosphere over Prolonged Solar Quiet Periods by ^{44}Ti Measurements in Meteorites

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The heliospheric magnetic field (HMF) is controlled by solar activity, as established by measurements over the last few decades, but its characteristics when the sun was quiet for prolonged periods, such as during Gleissberg or Maunder minima, are not known. Titanium-44, produced in meteorites, provides a monitor of the galactic cosmic ray (GCR) flux and allows estimation of the modulation effect of the sun for the period 1883 to 1992. The titanium-44 activity is consistent with the expected value, but the increase, due to the last Gleissberg minimum, is four times greater than expected for a GCR modulation based solely on sunspot numbers. This result implies that the HMF was weaker than at present and as a result the GCR flux (for energy greater than 1 gigaelectron volt) was higher between 2.2 to 3.6 protons per square centimeter per second per 4π steradians at 1 to 3 astronomical units in solar cycles 12 to 15.

Production of cosmogenic radioisotopes in meteorites depends on the GCR flux, which is controlled by the HMF which, in turn, depends on solar activity. GCR flux is inversely correlated with sunspot number. The radioisotopes can, therefore, serve as proxy records of solar activity. This approach has been used to understand solar behavior in the past based on records from terrestrial archives, such as ^{14}C in tree rings (*1*) and ^{10}Be in ice cores (*2*) and sediments, but such records are not free from interference of the terrestrial climate that governs the deposition rates of these isotopes in various terrestrial reservoirs (*1*). A similar approach, that of using isotopes produced in meteorites, offers a direct assessment of the solar activity, free from the influence of climatic cycles on Earth. The effect of the 11-year solar cycle has been quantitatively estimated by analysis of ^{22}Na (half-life, 2.6 years) in meteorites (*3*, *4*) and lunar samples (*5*). Here we use measurements of ^{44}Ti in stone meteorites with known dates of fall to assess century-scale variations in GCR flux.

^{44}Ti has a mean life of about 96 years (*6*) and is thus ideal for monitoring the behavior of the sun during the past 100 or 200 years. It is produced in cosmic-ray interactions (>70 MeV) in meteoritic iron and nickel (*7*). Attempts to determine its variation by radiochemical separation in several meteorites have not yielded measurable activity (*8*) because its production cross section is low, resulting in ^{44}Ti activity of about 1 dpm per kilogram of chondrite. We have therefore designed (*9*) a sensitive and selective γ-ray spectrometer to measure activity of its daughter, ^{44}Sc (half-life, 3.93 hours), which is in secular equilibrium with its parent, ^{44}Ti. ^{44}Sc decays by β^+ emission in coincidence with a 1157-keV γ-ray. Interference from the ubiquitous, naturally occurring ^{214}Bi (1155 keV) can be suppressed when the Ge detector is used in coincidence with a surrounding NaI(T) detector that operates only in annihilation γ-ray windows. By situating the spectrometer at an equivalent water depth of 70 m under Monte dei Cappuccini in Torino in a nitrogen atmosphere, a background of ~0.6 counts per day in 1157-keV channels was achieved.

We counted 10 fragments of nine chondrites: Alfianello (L6, 625 g), Lancon (H6, 1080 g), Holbrook (L6, 331 g), Olivenza (LL5, 247 g), Rio Negro (L4, 388 g), Monze (L6, 165 g), Dhajala (H3, 700 g), Torino (H6, 445 g), and Mbale (L5/6, 700 and 730 g) that fell during the period 1883 to 1992. The activity of ^{26}Al (1809 keV), ^{40}K (1461 keV), and ^{44}Ti (^{44}Sc, 1157 keV) was measured by counting fragments for a period

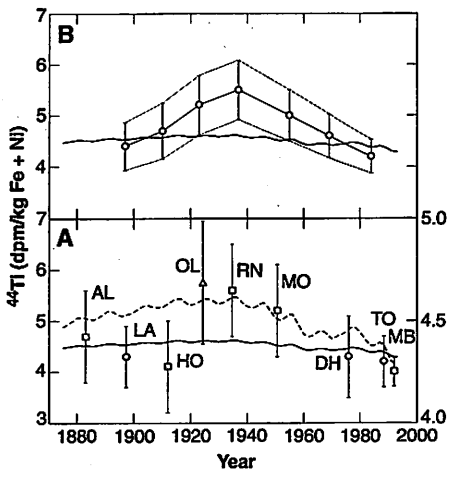

Fig. 1. (**A**) Observed ^{44}Ti activity (dpm per kilogram of Fe + Ni) for H (○), L (□), and LL (△) chondrites. AL, Alfianello; LA, Lancon; HO, Holbrook; OL, Olivenza; RN, Rio Negro; MO, Monze; DH, Dhajala T273; TO, Torino; MB, Mbale-T and Mbale-A. The variation expected due to modulation of GCR by sunspot activity is shown by a thick curve and, on a magnified (right hand) scale, by a dashed curve to illustrate small variations expected due to the Schwabe cycles. (**B**) Comparison of the three-point running mean and the error on the trend (dotted curve) with the expected profile based on the sunspot numbers.

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ranging between 4 to 12 weeks. The inherent ^{40}K concentration in the meteorites was used as an internal standard for determining the effective efficiency of counting for each meteorite fragment, as described (*10*). The measured activity of ^{44}Ti was corrected for decay to the time of fall (Table 1 and Fig. 1A).

To determine the cosmic ray flux responsible for production of the observed activity of ^{44}Ti, it is necessary to correct for (i) variation in target element (Fe and Ni) concentration and (ii) shielding depth within the meteorite. The shielding correction was made on the basis of the density of tracks produced by cosmic ray heavy nuclei (*11*), which were measured in several spot samples of each meteorite. Together with exposure ages, calculated from cosmogenic ^{21}Ne (*12*), the track density allowed us to determine the preatmospheric size (R_o) and shielding depths (Δx) of the meteorite fragments (*11*). Further constraints on the shielding depths were obtained by comparison of ^{26}Al activity with the production depth profiles (*13*). Measurements of ^{44}Ti in two fragments of Mbale (Δx = 12 and ~40 cm) indicated that the change in ^{44}Ti production rate with depth was small, as expected for high-energy products. The shielding depths of all the meteorite fragments were estimated to lie between a narrow range of 8 to 25 cm and, except for Olivenza and Dhajala, R_o of all meteorites was between 23 and 40 cm. Olivenza and Dhajala were larger bodies in space (estimated R_o, 85 and 110 cm, respectively). The shielding correction, normalized to Torino ($[R_o, \Delta x]$ = [24, 16] cm), was small in all meteorites except Dhajala and the Mbale (T) fragment, where it approached 10%.

The observed ^{44}Ti activity was compared with the expected value calculated from the isotope production model (*14*) (Fig. 1A). The model requires inputs of GCR flux, J_G (protons >1 GeV/cm^2·s·4π sr), and excitation functions of ^{44}Ti from Fe and Ni. $J_G(t)$, appropriately modulated by the solar activity, was calculated on the basis of sunspot numbers (Fig. 2A) and Climax neutron monitor count rates (N_m) as follows.

The relation between $N_m(t)$ and annual sunspot numbers, $R(t)$, for the period 1953 to 1992 is given by

$$N_m(t) = 4265.133 - 3.961559[R(t-1)] - 0.0116098[R(t-1)]^2 + 6.018108 \times 10^{-5}[R(t-1)]^3$$

This relation was extrapolated back to 1800. J_G was then calculated by a linear regression between the normalized (January 1965: 4291.7 counts/hour × 100 = 1) neutron monitor rate, N_m*, as given by

$$J_G = 8.13N_m^* - 5.845$$

as described (*10*). The flux, used with the isotope production model (*14*), gives the expected profile of ^{44}Ti as a function of time of fall (Figs. 1 and 2B). A systematic error may be present in the absolute production rate as a result of uncertainties inherent in the model and the excitation function, but the shape of its time profile depends only on the variation in sunspot numbers (Fig. 2A). The small oscillations in production rates (Fig. 2B) are due to the 11-year Schwabe cycle and the two large maxima are due to the Gleissberg minima, with a phase lag due to the integrating nature of the ^{44}Ti activity over its mean life.

The agreement between the observed ^{44}Ti activity and the expected values (Fig. 1A) is reasonably good, taking into account the errors of measurement (7 to 20%), and validates the procedure used, except that the activity in meteorites that fell during 1920 to 1950 appears to be higher. To ascertain whether the increase is real or a statistical fluctuation, and to reduce the standard deviations, we consider moving averages of the data. The three-point running mean together with the error band (Fig. 1B) shows a maximum around 1935, indicating that the trend of increased activity is significant. In order to highlight the variability of the data and the phase of the maximum, we show the production profile on a magnified (×4) scale (Fig. 1A, dashed curve). The phase of the observed maximum clearly agrees with that expected due

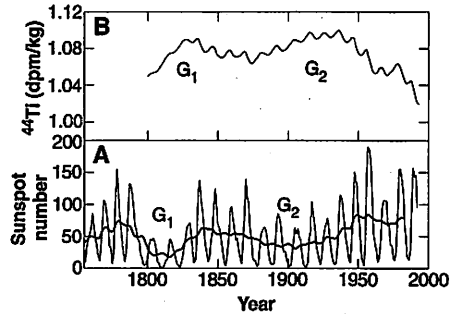

Fig. 2. (**A**) Annual mean sunspot numbers from cycle 1 to 22. The locations of Gleissberg minima of 1810 and 1910 are indicated by G_1 and G_2, respectively. The thick curve shows the 22-year running mean of the sunspot numbers. (**B**) The calculated production rate of ^{44}Ti activity as a function of time based on sunspot numbers given in (A). Curve shows the variation expected due to the 11-year Schwabe cycle and the 90-year Gleissberg cycle. The periods of peak activity expected due to Gleissberg minima G_1 and G_2 are indicated.

Table 1. Track density, calculated shielding depths, and ^{44}Ti activity at the time of meteorite fall. Time of fall is given as date.month.year. Ma, million years ago.

Meteorite (class)	Date of fall	Exposure age (Ma)	Track density (10^5/cm^2)*	Shielding (cm)*	Fe + Ni (%)	^{44}Ti†	
						a	b
Alfianello (L6)	16.2.1883	25.3	1.52 (O, 25)	11.5 ± 1	22.95	1.1 ± 0.2	4.7 ± 0.9
Lancon (H6)	20.6.1897	6.6	1.37 (O, 74)	11 ± 3	28.52	1.3 ± 0.2	4.3 ± 0.6
			4.56 (O, 107)	12.5 ± 2.5			
Holbrook (L6)	19.7.1912	16	0.5 (P, 51)	17 ± 2.5	21.6	0.8 ± 0.2	3.8 ± 0.9
			0.8 (P, 15)	12.5 ± 1.5			
			5.0 (P, NR)	9.5 ± 1.5			
Olivenza (LL5)	19.6.1924	11.6	<1	14.5 ± 2.5	20.26	1.2 ± 0.2	5.8 ± 1.2
Rio Negro (L4)	21.9.1934	21.5	0.42 to 5.4 (O, 10 to 78)‡	14 ± 6	22.57	1.3 ± 0.2	5.6 ± 0.9
			1 to 2.59 (P, 22 to 38)‡				
Monze (L6)	5.10.1950	9.9	0.69 (O, 9)	11.6 ± 0.8	22.0	1.1 ± 0.2	5.2 ± 0.9
			0.93 (P, NR)	14.5 ± 2.5			
Dhajala (H3)	28.1.1976	8.9	0.0096§	24	28.74	1.2 ± 0.3	4.4 ± 0.8
Torino (H6)‖	18.5.1988	68	4.2	16 ± 2	27.26	1.2 ± 0.2	4.2 ± 0.5
Mbale-A (L5/6)	14.8.1992	26.9	2.14 to 3.6 (O, 199)	11.6 ± 0.8	22.9	1.0 ± 0.1	4.0 ± 0.4
Mbale-T			<0.2	~40		0.8 ± 0.1	4.0 ± 0.4

*O, Olivines; P, Pyroxenes; number of tracks counted are given in parentheses [NR, not recorded (*11*)]. Mean shielding depth ± range for different fragments was calculated from (*11*). †^{44}Ti activity (a) dpm per kilogram of meteorite, uncorrected for target abundance and shielding; and (b), dpm per kilogram of (Fe + Ni), corrected for shielding and normalized to Torino. ‡Rio Negro has solar flare tracks (*9*), which are not included here. §Data are from (*16*). ‖Track data are from (*10*). Torino had a complex exposure: a long exposure (stage I) and a short exposure (≤10 Ma, stage II). Composite exposure age of 68 Ma was recalculated from (*10*).

to the Gleissberg minimum early in this century. However, the observed increase of about 20% is four times that expected based on the sunspot numbers.

The observed high ^{44}Ti and the inferred high GCR flux during the Gleissberg minimum suggest a much-weakened modulation of GCR during prolonged solar quiet periods. The heliosphere, during the 11-year solar cycles, is divided into two hemispheres of opposite polarity by a wavy heliospheric neutral sheet (HNS) whose inclination to the sun's rotational equator increases from a few degrees at solar minimum to >70° at solar maximum and whose undulations also increase with solar activity (*15*). The HMF is relatively smooth during the solar minima. The enhanced GCR fluxes required for the higher production of ^{44}Ti can be achieved if (i) the HNS becomes smooth and remains close to the helioequator; (ii) the HMF becomes weak, more regular, and orderly; or (iii) the size of the heliosphere shrinks during a prolonged period of low sunspot numbers, so that a larger flux of cosmic rays can enter into the inner heliosphere. The GCR flux, J_G(>1 GeV), near 1 AU during maximum and minimum of the 11-year solar cycle (*14*, *16*) in the past four Schwabe cycles, varied between 1.4 and 2.2 to 2.5 protons/cm^2·s·4π sr, with an average value of 1.7 protons/cm^2·s·4π sr. The observed activity can be explained if J_G varied between 2.2 and 3.6 protons/cm^2·s·4π sr at 1 to 3 AU during solar cycles 12 to 15 covering the Gleissberg minimum at the turn of the century. Such a regularity of HMF may also justify the even higher GCR fluxes estimated for the Maunder minimum, when sunspot numbers were low for about 70 years (1645 to 1715), and can explain the high activities of ^{14}C and ^{10}Be observed on Earth (*17*).

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Particle Formation in the Upper Tropical Troposphere: A Source of Nuclei for the Stratospheric Aerosol

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Atmospheric measurements and numerical calculations described here indicate that binary homogeneous nucleation of H_2SO_4-H_2O particles occurs in the upper tropical troposphere. Particle concentrations decrease with increasing altitude above the tropical tropopause as a result of coagulation during the upward air transport produced by stratospheric circulations. During the extended periods of time that volcanic eruptions do not strongly influence stratospheric particle number concentrations, particles formed in the upper tropical troposphere provide nuclei upon which oxidized sulfur gases condense in the stratosphere. This particle source, coupled with aerosol microphysical properties and atmospheric transport, governs the number concentration of particles in the lower tropical and mid-latitude stratosphere.

The stratospheric aerosol layer, or Junge layer, is a fine mist of highly concentrated liquid sulfuric acid (H_2SO_4) particles that envelops the Earth at altitudes from a few kilometers above the tropopause (~9 to 17 km) to ~28 km. The particles are typically 0.1 to 0.3 μm in diameter and are present at number mixing ratios (NMR, number of particles per milligram of air) of ~60 mg^{-1} (*1–3*). These stratospheric H_2SO_4-H_2O particles provide sites for heterogeneous reactions that repartition nitrogen species, ultimately leading to reduced ozone concentrations (*4*, *5*). In the polar regions, reactions on these particles may also activate chlorine, which catalytically destroys ozone (*6*). In both of these cases, the surface area provided by the particles for the heterogeneous reactions is an important parameter in the chemical process. In the polar vortices, H_2SO_4-H_2O particles are the nuclei on which polar stratospheric clouds (PSCs) form (*7*). Changes in the concentration of PSC nuclei may change the concentration of PSC particles, the surface area available for chlorine activation, and the process of particle sedimentation that leads to denitrification and polar ozone depletion (*8*).

The surface area available for heterogeneous reactions in the stratosphere depends largely on the number of particles upon which the total particle mass is distributed. Most of the mass of the stratospheric aerosol layer originates from reduced sulfur gases (OCS, CS_2, and SO_2) transported from the troposphere (*2*). These gases oxidize in the stratosphere to H_2SO_4, which then condenses onto preexisting particles (nuclei). In volcanically quiescent periods, OCS is believed to be the dominant source of stratospheric H_2SO_4 mass (*9*). The sources of the nuclei on which the H_2SO_4 condenses are poorly known. Many particles form in the stratosphere from homogeneous nucleation of H_2SO_4-H_2O after major volcanic injections of SO_2 (*10*, *11*). However, within ~6 months after such eruptions, stratospheric NMRs decay to near prevolcanic values (*12*). When volcanic effects are small, the stratosphere's degree of supersaturation with H_2SO_4 vapor is insufficient for particle formation (*3*, *10*, *13*), with some minor exceptions (*14*). These findings im-

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ply that there are sources of particles outside of the stratosphere that provide the nuclei for maintenance of the Junge layer during volcanically quiescent periods, which often extend for several years (*3*).

Possible sources of stratospheric nuclei include condensed meteoritic vapor (*15*) and particles transported from the troposphere. Measurements of particle composition (*16*) and consideration of the upwelling air motions in the tropical stratosphere (*17*) provide evidence against a significant meteoritic component in particles below an altitude of 25 km. Although some studies have suggested that the troposphere could provide a source of nuclei for the stratospheric aerosol (*1, 2, 10, 18*), there is only limited observational evidence for tropospheric particles entering the lower stratosphere (*1, 19*).

The measurements analyzed here were obtained in the mid-latitudes, polar regions, and tropics in both hemispheres between 1987 and 1994 (*20*). We excluded data known to be affected by volcanic eruptions and by particles formed in the polar vortices (*14*). Measurements made in fall, winter, and spring dominate the extratropical data. Condensation nucleus counters (CNCs) measuring the NMR of particles in the diameter (D_p) range of 0.008 to ~3 μm (*21*) were flown on NASA U-2 and ER-2 aircraft to altitudes of ~21 km. Because the stratospheric aerosol population is usually dominated by particles in this size range, the total particle NMR was well represented by the measurements. In 1993 and 1994, a second CNC measured nonvolatile-particle NMR by heating the aerosol stream to 192°C before counting.

Every profile that penetrated the tropopause was classified as tropical or extratropical on the basis of an analysis of chemical-tracer and meteorological data (*22*). The data were grouped into intervals of potential temperature θ, and the medians and interquartile ranges were calculated (Fig. 1). Potential temperature is the temperature that an air parcel would have were it compressed or expanded adiabatically from the local pressure to 10^5 Pa. Horizontal air motions in the stratosphere approximately follow constant-θ surfaces over time scales of a few weeks.

A maximum in particle NMR is present near the tropopause in both tropical and extratropical cases. This maximum is about seven times larger inside the tropics than outside. Particle NMRs decrease smoothly with θ above the tropical tropopause, reaching extratropical values near 470 K. The relative variability of the extratropical NMRs is considerably larger than that for tropical values between the tropopause and 440 K. High particle NMRs were found on two occasions in the upper troposphere outside of the tropics; thus, the processes that lead to the high particle NMRs are not unique to the tropics but are more common and widespread there.

Two pieces of evidence suggest that the particles observed near the tropical tropopause are composed of H_2SO_4-H_2O droplets. Laboratory studies show that the heated inlet of the CNC used in 1993 and 1994 is effective at volatilizing >90% of all H_2SO_4-H_2O particles with $D_p < 0.04$ μm. Near the maximum in particle mixing ratio just below the tropical tropopause, >95% of the particles were volatile at 192°C (Fig. 2A), consistent with the particles being composed of H_2SO_4-H_2O. Electron microscope analysis of individual particles collected by an impactor (*16*) just below the tropical tropopause at 20°N on 3 May 1993 showed the particles to be nearly monodisperse with diameters of ~0.015 μm and to contain sulfur as the only element significantly above background (*23*). Given these observations and the well-documented presence of sulfur gases and H_2SO_4 particles in the free troposphere (*16, 24*), we conclude that a particle composition of H_2SO_4-H_2O is likely.

Near-tropopause measurements of particle NMRs taken at extratropical locations contained a much smaller proportion of volatile particles (Fig. 2B). The likely cause of the reduced particle volatility is incorporation over time of small amounts of ammonia into the particles to form partially neutralized sulfate particles, which the heated inlet on the CNC is unable to volatilize. Thus, low concentrations of nonvolatile particles near the mid-latitude tropopause probably indicate that the particles were not recently formed by homogeneous nucleation of H_2SO_4 and H_2O.

The above measurements suggest that particles composed of H_2SO_4 and H_2O nucleate near the tropical tropopause. We have evaluated the formation rate of H_2SO_4-H_2O particles using a numerical model of homogeneous bimolecular nucleation (*25*). This model simulates nucleation rates under conditions in which the pure substances (H_2O and H_2SO_4) are subsaturated. Because of well-known uncertainties in the classical nucleation theory used for this calculation (*26*), we use the results as a qualitative indication that nucleation is likely or unlikely to occur, rather than as a quantitative estimate of particle production rates.

Vertical profiles of temperature, pressure, and H_2O mixing ratio measured aboard the ER-2 (*27*) were used as input to the numerical model. The mixing ratio of H_2SO_4 was estimated from a one-dimensional numerical model of the stratospheric aerosol (*28*). The height of the local tropopause was determined from in situ measurements and from a remote-sensing microwave temperature profiler on board the ER-2 (*29*). Particle nucle-

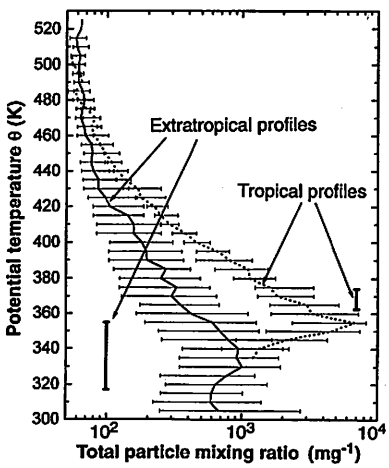

Fig. 1. Vertical profiles of particle NMR measured outside of the tropics (solid line) and within the tropics (dashed line), as defined by meteorological analysis and tracer relations. Data from each profile that penetrated the tropopause were binned into 5 K increments of θ, and the median values (lines) and interquartile ranges (containing the middle 50% of the data, shown as error bars) of these binned values were calculated. All profiles are thus weighted equally. The range of potential temperatures enclosing the tropopause heights for each data set are indicated by vertical bars.

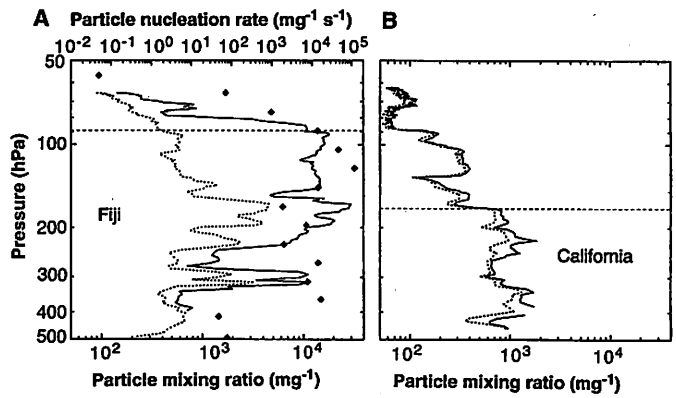

Fig. 2. Vertical profiles of total particle mixing ratio (solid line) and nonvolatile particle mixing ratio (dotted line). Volatile particles (the difference between the lines) are interpreted as being mostly H_2SO_4-H_2O. The approximate height of the tropopause is shown by the horizontal dashed lines. (**A**) Profile measured in tropical air during an ascent from Nadi, Fiji, on 29 March 1994. The formation rate of H_2SO_4-H_2O particles calculated from a binary homogeneous nucleation model with input data measured during this profile is shown by the symbols. (**B**) Profile measured in mid-latitude air during an ascent from northern California on 14 May 1993.

ation rates (in particles per milligram of air per second) as a function of altitude were calculated for the ascent from Fiji on 29 March 1994 (Fig. 2A). The model results indicate a relative tendency for nucleation in the upper troposphere, with a maximum nucleation rate at a pressure of 120 hPa, about 2 km below the tropopause. The shape of the nucleation rate profile is qualitatively similar to that of the measured particle NMR profile. The measurements show a rapid decrease in particle NMR just above the tropopause, suggesting that particle formation is not important in this region.

The results of the nucleation modeling support the observational evidence that new H_2SO_4-H_2O particles are formed by homogeneous binary gas-to-particle conversion in the upper tropical troposphere. This maximum in particle NMR lies in a region of net upward mass transport of air through the tropical tropopause and into the middle stratosphere (*17*). This net upwelling is a component of the residual circulation of the stratosphere. The mechanisms of cross-tropopause transport are poorly understood but probably involve penetration of the lower stratosphere by convective clouds in the tropics (*30*); small particles may survive such cloud processing (*31*). Regardless of the method of transport, our data suggest that particles of upper tropospheric origin are frequently found above the tropical tropopause.

Once particles have reached the lowest regions of the stratosphere in the tropics, they are advected horizontally and vertically. Mean vertical air velocities in the stratosphere have been estimated recently from global meteorological and chemical data coupled with radiative transfer calculations (*17*) and can be derived from satellite measurements of water vapor (*32*). Vertical velocities within the lower tropical stratosphere are $\sim 2 \times 10^{-4}$ m s^{-1}, with higher velocities near the tropopause (*33*). Small particles in the lower tropical stratosphere have negligible gravitational sedimentation velocities and should also experience a net upward motion of this magnitude. Because the particles undergo significant diffusive movement, they may collide and coalesce during transport, which reduces NMR at a rate roughly proportional to the square of the particle concentration.

It has been asserted that steep gradients in the altitude profiles of particle NMRs in the tropics are inconsistent with a tropical tropospheric source for stratospheric nuclei (*34*). When the vertical axis is transposed from altitude to θ (Fig. 1), the observed average gradient in total particle NMR with increasing altitude is relatively gradual and monotonic above the tropical tropopause. We used a coagulation model to examine if this profile is consistent with coagulational depletion of particle number during ascent away from a near-tropopause source. An air parcel was initialized at 370 K (which is within the range of potential temperatures encompassing the observed tropopauses) and advected vertically from 370 to ~480 K with mean tropical velocities derived from calculations of the stratospheric residual circulation (*17*). Particle NMR was calculated as a function of θ by a detailed model of particle coagulation (*35*). Particle NMRs measured at 370 K and particle sizes estimated from the measurements were used as input, and mixing of aerosol with mid-latitude characteristics into the tropical parcel was simulated (*36*).

Nominal values of the input parameters result in particle NMRs that very closely match the median tropical profile (Fig. 3). The results are sensitive to the assumed particle diameter and to the vertical velocity profile and less sensitive to mixing of the tropical parcel with mid-latitude air.

There are several important implications of the coagulation model results. First, nucleation of particles above the troposphere is not required in the model to match the observations. The particle NMRs observed at $\theta > 475$ K (~20 km) can be explained purely by an upper tropospheric particle source and upward transport with horizontal mixing. Second, coagulation rates become very slow at the low particle NMRs found near the top of the profile: particle NMR half-lives for coagulation exceed 1 year. Thus, the particle NMR observed near 20 km in the tropics can account for the particle NMRs observed throughout the stratospheric sulfate layer, even over long transport times. Third, to accurately simulate the observed profile of particle mixing ratio, one must incorporate both horizontal mixing and the vertical variation in vertical velocity. A quantitative understanding of atmospheric motions, as well as of particle microphysics, is necessary in order to understand the spatial distribution of particle number and surface area in the stratosphere. Fourth, several statistically robust studies of stratospheric particle composition (*16*, *37*) have not found the diversity of particle compositions typical of the mid-latitude free troposphere (*16*). However, homogeneous nucleation of H_2SO_4-H_2O particles near the tropical tropopause, followed by vertical transport to the lower tropical stratosphere, would explain the relative homogeneity of stratospheric particle composition observed.

Finally, profiles of particle NMR in the mid-latitude lower stratosphere often show regions of enhanced values above the tropopause. These enhanced particle NMRs appear in the averaged mid-latitude profile in Fig. 1 as a large relative interquartile range and minor layering in the median values. Because vertical transport is extremely restricted in the lower stratosphere in mid-latitudes (*38*), the mid-latitude troposphere is unlikely to be the source of these layers. A profile measured during descent above northern California shows the magnitude of the layering (Fig. 4). The maxima in the particle NMR profile lie between the median extratropical and tropical profiles. Measure-

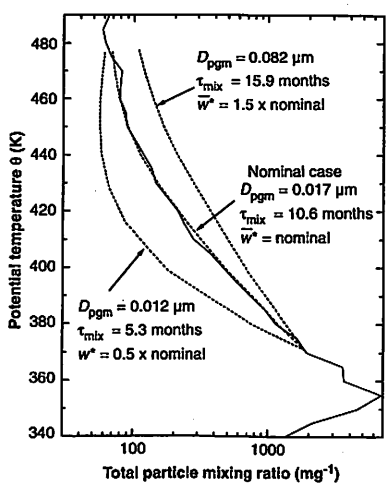

Fig. 3. Total particle mixing ratios calculated with a numerical model of particle coagulation and mixing in an air parcel ascending in the tropics. Dashed lines are model output for the nominal case and for the extreme cases from sensitivity tests. The model runs were initiated at θ = 370 K, within the range of tropical tropopause potential temperatures. The nominal residual mean vertical velocity $\bar{w}^*$ varied with altitude, falling from $\sim 8 \times 10^{-4}$ m s^{-1} at 370 K to $\sim 2 \times 10^{-4}$ m s^{-1} at 475 K (*33*). For sensitivity tests, extremes of possible geometric mass median diameter (D_{pgm}) were determined from the response characteristics of the CNC and optical particle-size spectrometers aboard the ER-2. The time scale for mixing of mid-latitude air into the tropical parcel, τ_{mix}, was estimated from tracer relationships (*41*). The profile of the tropical median total particle mixing ratio from Fig. 1 is given by the solid line.

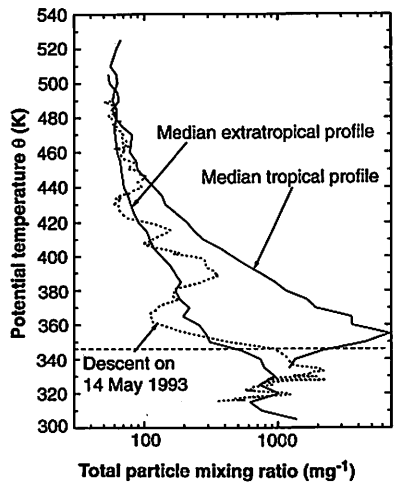

Fig. 4. Vertical profile of total particle mixing ratio from a descent over northern California on 14 May 1994. The median extratropical and tropical mixing ratio profiles from Fig. 1B are shown for comparison. The approximate tropopause level is indicated with a horizontal dashed line.

ments of gas-phase species used as tracers (*39*) indicate that the layers had been transported from the tropics. Such layered profiles are typical of the lower mid-latitude stratosphere in fall, winter, and spring (our summertime data are too few to allow general interpretation). Other analyses of tracer measurements have shown that quasi-horizontal transport from the near-tropopause region of the tropics occurs rapidly in fall, winter, and spring to the mid-latitudes between the tropopause and altitudes of at least 19 km (*38*, *40*). Together, these observations indicate that particles produced in the upper tropical troposphere are frequently transported to the lower mid-latitude stratosphere, where they may significantly affect particle number and surface area mixing ratios.

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Transcription Against an Applied Force

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The force produced by a single molecule of *Escherichia coli* RNA polymerase during transcription was measured optically. Polymerase immobilized on a surface was used to transcribe a DNA template attached to a polystyrene bead 0.5 micrometer in diameter. The bead position was measured by interferometry while a force opposing translocation of the polymerase along the DNA was applied with an optical trap. At saturating nucleoside triphosphate concentrations, polymerase molecules stalled reversibly at a mean applied force estimated to be 14 piconewtons. This force is substantially larger than those measured for the cytoskeletal motors kinesin and myosin and exceeds mechanical loads that are estimated to oppose transcriptional elongation in vivo. The data are consistent with efficient conversion of the free energy liberated by RNA synthesis into mechanical work.

RNA polymerases play a critical role in gene expression by synthesizing RNA transcripts containing genetic information copied from DNA templates. The nascent RNA chain is elongated in a chemical reaction during which appropriate ribonucleoside triphosphates (NTPs) are condensed with the RNA 3′ end and pyrophosphate anions (PP_i) are released. RNA polymerases move along DNA while copying it, advancing on average a distance of 1 base pair (bp) (~0.34 nm along the DNA helix axis) for each nucleotide added to the transcript [(*1*) but see also (*2*, *3*)]. In vivo, this movement performs mechanical work against hydrodynamic drag and other external forces (*4*, *5*) and therefore requires a source of free energy. The energy is provided by the condensation reaction itself, which is energetically favorable at physiological NTP and PP_i concentrations (*1*). Thus, RNA polymerases may be viewed as molecular motors that catalyze a biosynthetic reaction while using a portion of the excess free energy from the reaction to perform mechanical work. Although translocation of RNA polymerase molecules along DNA has been detected by microscopy (*6*–*8*), little is known about the ability of these enzymes to move against opposing forces or about their energy conversion effi-

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ciencies. We report here observations of the movement of single *E. coli* RNA polymerase molecules along DNA templates. The measurements were made with a microscope-based optical trapping interferometer (*9*). Calibrated forces were applied in the direction opposing translocation by the polymerase to a small spherical particle (a polystyrene bead) attached to the DNA, while the position of the particle was simultaneously measured. When a sufficiently high force was applied, movement by the enzyme could be stalled, in many cases reversibly. Our results show that this nucleic acid polymerase is a powerful biological motor that can exert considerable force and may operate with energy conversion efficiencies comparable to those of prototypical mechanoenzymes, such as myosin and kinesin.

We conducted experiments using an assay developed (*6*, *7*) to study transcriptional elongation by single molecules of *E. coli* RNA polymerase in vitro (*10*). Ternary transcription complexes consisting of single molecules of RNA polymerase associated with a DNA template and a nascent RNA transcript were assembled in solution, halted by NTP depletion, and adsorbed onto the cover glass surface of a microscope flow cell. Polystyrene beads (0.52 μm in diameter) were attached to the transcriptionally downstream ends of the DNA molecules so that each bead became tethered to the surface by its connection through the DNA and the polymerase. When supplied with NTPs, up to half of the immobilized transcription complexes are enzymatically active; bead-labeled complexes display elongation kinetics indistinguishable from those of unlabeled complexes in solution (*6*, *7*). In a typical experiment, a transcription complex was first located by video-enhanced, differential interference contrast light microscopy. The complex was identified visually by the Brownian motion of the bead, which was constrained by the length of its DNA tether to a small region centered over the attachment position of the polymerase (*6*, *7*). For the studies described here, the microscope was equipped with an optical trapping interferometer ("optical tweezers" plus a position sensor) that could exert calibrated forces up to ~100 pN on a bead while simultaneously measuring its displacement with subnanometer precision and millisecond time resolution (*9*, *11*). With the laser light shuttered, the optical trap was moved to the region near the bead. The shutter was then opened to activate the trap, and the bead was captured. By adjusting the trap controls, we positioned the center of the bead directly over the polymerase, typically at a height of ~590 nm above the cover glass surface (this particular height was chosen to bring the bead as close as possible to the surface without the risk of touching during subsequent measurements). We then repositioned the trap by moving it at a constant height along the direction of greatest detector sensitivity [the direction defined by the shear axis of the Wollaston prism; see (*9*, *12*)] until the DNA straightened and was held under very light tension, with the bead displaced just 30 to 70 nm from the trap center (Fig. 1A).

We started transcriptional elongation of bead-labeled complexes by exchanging the buffer inside the flow cell with one containing NTPs. In most experiments, elongation was begun immediately before establishment of the initial configuration just described. During elongation, the template was pulled by the stationary polymerase molecule, developing further tension in the DNA between the bead and the polymerase and drawing the bead away from the trap center (Fig. 1B). The optical trap acts as a nearly linear spring of stiffness α_{trap} attached to a stationary reference frame and exerts a force F_{trap} on the bead (Fig. 1C). The bead adopts a position where F_{trap} is balanced by the force F_{tc} exerted by the polymerase, acting through the DNA. The series elasticity due to the DNA, polymerase, and associated linkages acts as a spring of stiffness α_{tc}. By calibrating the optical trap stiffness and determining the displacement of the bead, we could measure F_{trap} and thus F_{tc}. The force could be determined without knowledge of the stiffness α_{tc}, which depends on the length of DNA as well as the applied force (*13*).

During control experiments in which no NTPs were added, beads remained at an approximately fixed distance from the trap center. Slight changes seen in the interferometer signal (mean rate ~0.3 nm s^{-1} toward the center of the trap) were attributable to instrumental drift.

In elongation experiments using high concentrations of NTPs (1 mM each of adenosine triphosphate, guanosine triphosphate, cytidine 5′-triphosphate, and uridine 5′-triphosphate), 1 μM PP_i, and low trapping force (laser power at specimen, 25 mW; trap stiffness α_{trap}, ~0.03 pN nm^{-1}) (*14*), we observed continuous movement of beads out to the limit of the usable range of the trap, located roughly 200 nm from the trap center. Once a bead reached this limit, the trap was manually repositioned to bring the bead closer to the trap center so that observation could continue (Fig. 2A). In five of seven elongating complexes studied in this way, beads moved continuously to the limit without stopping, moving at similar velocities after the trap was repositioned. (One complex stopped and failed to restart; another stopped for ~18 s and then continued elongation to the trap limit.) The bead velocity, 4.3 ± 1.3 nm s^{-1} (mean ± SD) (*15*), was comparable to elongation rates measured under similar conditions in solution (4.4 to 6.8 nm s^{-1}) (*6*) and in previous microscope experiments (4.2 ± 1.7 nm s^{-1}) (*7*).

Different behavior was observed when higher trapping forces were used (laser power at specimen, 82 to 107 mW; α_{trap}, ~0.09 to 0.12 pN nm^{-1}). In this regime, 66 of 77 elongating complexes (86%) stopped translocating (stalled) once the bead encountered the high-force region of the trap (Fig. 2, B and C) (*16*, *17*). To test whether stalling was reversible, we maintained the bead at stall, typically for 10 to 15 s, then repositioned the trap center closer to the bead, reducing F_{trap}. After force reduction, 24 of the 66 stalled complexes resumed movement, and some complexes could even be stalled multiple times this way (Fig. 2C). After recovery from stall, the velocity of bead movement in the low-force region of the trap was similar to that

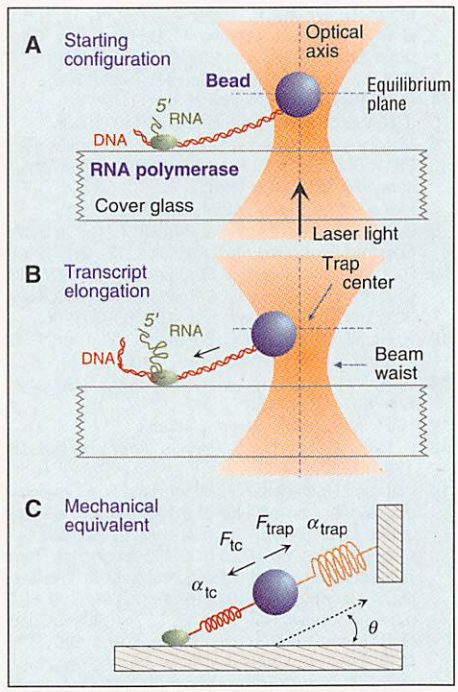

Fig. 1. A cartoon illustrating essential features of the experiment (not to scale). (**A**) The configuration at the start of transcriptional elongation. An RNA polymerase molecule (green ellipsoid) is attached to the cover glass surface of a flow cell. The polymerase is bound to template DNA (red) and has begun to synthesize a transcript RNA (green). A polystyrene bead (blue sphere) is attached to the downstream end of the DNA and is captured and held under slight tension by the light of the optical trap (pink). (**B**) The configuration during subsequent transcriptional elongation. The trap center is located on the optical axis but slightly above the narrow waist of the focused laser beam. The polymerase has proceeded for some distance along the DNA, shortening the segment between the bead and the polymerase. The bead is pulled away from the trap center, increasing the restoring force of the trap. (**C**) The mechanical equivalent of the experimental geometry shown in (A) and (B). All components lie centered in a vertical plane oriented parallel to the Wollaston shear axis.

before stall. The remaining fraction of RNA polymerase molecules stalled irreversibly in that they did not resume elongation when F_{trap} was reduced. Irreversible stalling may be attributable to one or more of several possible causes: The polymerase may have been directly inactivated by the mechanical load, have suffered photodamage from the laser light, or have spontaneously converted into an inactive species ("transcriptional arrest") similar to that formed during transcriptional stalling induced by NTP depletion (*18*, *19*).

The simplest physical interpretation of reversible stalling is that it corresponds to the situation in which F_{trap} has increased to a level where it balances the maximal force that the polymerase can exert (F_{stall}), and no further progress is made. When F_{trap} is reduced, enzyme activity resumes (*20*). During both reversible and irreversible stalls, movement sometimes slowed gradually during the approach to F_{stall} (for example, the third stall in Fig. 2C), whereas in other cases movement continued at nearly constant velocity, slowing abruptly close to F_{stall} (for example, the first and second stalls in Fig. 2C). Transcription complexes display differing biochemical properties depending on the nucleotide sequence of the DNA to which they are bound (*21*). Abrupt reversible stalling may correspond to the arrival of the enzyme at a template position for which F_{stall} is somewhat lower than that of the preceding positions, producing rapid arrest. This interpretation also could explain why F_{stall} values determined for multiple stalls of a single complex often differed by more than the experimental uncertainty in measurement (*22*). It is tempting to speculate that some sites of abrupt stalling might correspond to DNA sequences that trigger cycles of discontinuous elongation (*2*, *3*) or "jumping." At such sequences, a portion of the RNA polymerase molecule may, during a single nucleotide addition cycle, move along the DNA by ~10 bp, an axial distance of ~3.4 nm (*23*). These large movements of the enzyme would be expected, all else being equal, to give stall forces significantly lower than those of single base pair movements, because the free energy available from nucleotide addition would be applied over a larger distance. Nucleotide sequence effects on behavior under mechanical load could be studied in future experiments through the use of DNA templates containing homopolymer tracts or direct repeats.

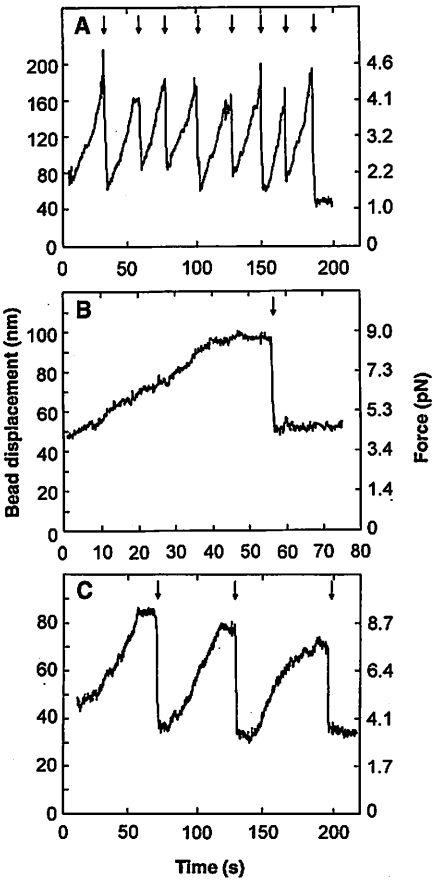

Fig. 2. Time courses for displacement from the trap center and the corresponding optical force applied to single beads driven by translocation of an RNA polymerase molecule along DNA. Force scales (right-hand axes) are nonlinear (*14*). The zero of the time axis is arbitrary. Reactions were conducted at 1 mM NTPs with 0.001 mM (**A**) or 1 mM (**B** and **C**) PP_i at laser powers of 25 (A), 82 (B), or 99 (C) mW. Vertical arrows designate times at which the trap was repositioned to reduce the optical force. An irreversible stall is shown in (B), and reversible stalls (first two stalls) and an irreversible stall (third stall) are shown in (C).

The distributions of F_{stall} values were obtained at 1 mM NTPs with 1 μM PP_i. The distributions of reversible and irreversible stall forces were statistically indistinguishable, with values of 13.0 ± 4.0 pN (mean ± SD, n = 8) and 12.6 ± 3.5 pN (n = 14), respectively. A possible explanation for the similarity of the distributions is that the reversibly stalled state is a precursor of the irreversibly stalled state. In this view, most or all of the stalled complexes are initially stalled reversibly, but a fraction of these are subsequently inactivated and therefore fail to resume elongation when F_{trap} is reduced.

F_{stall} was also determined at two higher concentrations of PP_i (0.5 and 1 mM) with 1 mM NTPs. Increasing the PP_i concentration to 1 mM slowed transcriptional elongation by about twofold (*24*), but F_{stall} remained nearly constant [at 0.5 mM PP_i, 13.0 ± 4.1 pN (n = 5) for reversible and 13.1 ± 2.6 pN (n = 10) for irreversible stalls; at 1.0 mM PP_i, 11.5 ± 2.9 pN (n = 11) for reversible and 9.9 ± 3.4 pN (n = 18) for irreversible stalls]; this observation may place important constraints on the RNA polymerase force-generation mechanism. In light of this result, stalls observed at all PP_i concentrations were pooled (Fig. 3A) to generate a global distribution with mean F_{stall} = 12.3 ± 3.5 pN (n = 24) for all reversible stalls. We previously found that single immobilized transcription complexes exhibit a range of velocities (*7*). Such heterogeneity among complexes may also contribute to the width of F_{stall} distributions. The polymerase is fixed to the glass at random and presumably is not free to rotate. Different spatial orientations of the polymerase with respect to the direction of the applied force might also cause F_{stall} to vary from molecule to molecule. A small fraction of the complexes did not stall before reaching the limit of the usable range of the trap (Fig. 3B). The assumption that these complexes would stall at a force larger than the maximum measurable force at the laser power used yields a lower limit estimate of 13.6 pN for the mean reversible F_{stall} (*25*). Despite systematic instrumentation errors for these measurements estimated at ~30% (*22*), the F_{stall} values for single RNA polymerase molecules are clearly much larger than are forces previously measured for single molecules of other mechanoenzymes: up to 6 pN for kinesin (*26*) and 3 to 5 pN for myosin (*27*).

The efficiency of chemomechanical energy conversion in RNA polymerase may be defined as the fraction of the total free energy change of the chemical RNA polymerization reaction ($\Delta G'_{polym}$) that the enzyme expends to perform mechanical work against an external load. Thermodynamically reversible motors are most efficient as they approach the point of stalling. The free energy available from the chemical reaction varies with the PP_i-to-NTP concentration ratio because these are the product and

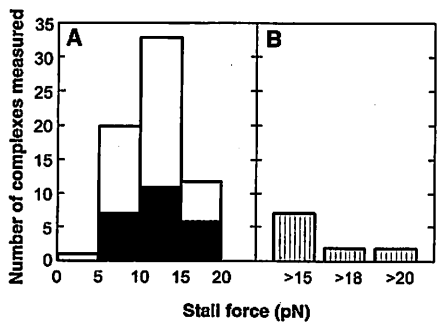

Fig. 3. Stall force distributions measured from pooled data obtained at 1 mM NTPs with 0.001, 0.5, and 1.0 mM PP_i. For beads exhibiting reversible stalls, only data from the first stall were included. Laser powers (and corresponding maximum measurable forces) of 82 mW (15 pN), 99 mW (18 pN), and 107 mW (20 pN) were used in 25, 33, and 19 measurements, respectively. (**A**) Stacked histogram of stall forces for irreversible (open portion of bars, n = 42) and reversible (solid portion of bars, n = 24) stalls. (**B**) Histogram of estimated lower bound of stall forces for complexes (n = 11) that did not stall before exiting the calibrated range of the trap. The number below each bar represents the maximum measurable force for the laser power used.

reactant species, respectively. For the conditions used, $\Delta G'_{polym}$ ranged from −7.2 kcal mol^{-1} (at 1 μM PP_i) to −3.1 kcal mol^{-1} (at 1 μM PP_i) (*1*). Under the assumption that each NTP consumed advances the polymerase 1 bp on average along the DNA (0.34 nm along the DNA axis), the mean F_{stall} estimate corresponds to maximal energy conversion efficiencies of 9, 19, and 22% at 0.001, 0.5, and 1 mM PP_i, respectively. These efficiencies are comparable to values obtained for biological motors such as kinesin [40 to 60% (*11*, *28*)] or myosin [12 to 42% (*27*)] (*29*).

Nucleic acid polymerases carry out biosynthetic reactions and are not ordinarily classified as mechanoenzymes. Nevertheless, our results indicate that individual molecules of *E. coli* RNA polymerase exert forces and operate with energy conversion efficiencies that are similar to those of prototypical mechanoenzymes, whose specialized function is to generate biologically useful force and motion. Inside living cells, interactions between transcription complexes and cellular structures or DNA-bound proteins create substantial forces that oppose the translocation of polymerases relative to DNA (*4*, *5*). For example, to function in opposition to loads imposed by transcription-induced supercoiling of plasmid DNA in vivo, *E. coli* RNA polymerase must generate forces estimated at ~6 pN (*30*). Forces of this magnitude are sufficient, for example, to stall solitary molecules of kinesin or myosin, but are nonetheless smaller than the forces achieved by RNA polymerase molecules in vitro. We anticipate that further development of optical measurements on single transcription complexes will allow detailed characterization of the multiple mechanical processes (*2*, *3*, *23*) by which RNA polymerase moves along DNA.

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14. We calibrated trap stiffness (α_{trap}) using free beads, either by measuring the power spectrum of the Brownian motion or by measuring the bead displacement from the trap center produced by viscous drag forces (*9*, *11*, *12*). Stiffnesses obtained with the use of these two methods differed by <10% for excursions below half the usable detector range (<100 nm). For larger excursions, the drag force method was used exclusively. Calibration was accomplished in two stages. First, the relation between digitized interferometer output voltage and true displacement (in nanometers) was established as described previously (*9*). Second, the relation between trapping force (in piconewtons) and displacement (in nanometers) was determined by the drag force method, with viscous forces being corrected for the proximity of the cover glass surface (*12*). The two data sets were then combined. In contrast to the case of silica particles (*11*), the force-displacement relation for polystyrene beads has significant nonlinearities in the outermost part of the trap; therefore, calibration data out to 150 nm were fit by a fifth-order polynomial to extrapolate interferometer signals into the region between 150 nm and ~200 nm. This procedure may miscalculate the actual force produced in the region beyond 150 nm; however, exclusion from the analysis of data taken in this region did not significantly alter the mean stall forces reported here. The estimated force at 200 nm displacement was taken to be the largest measurable force at a given laser power.
15. A correction for the contribution of tether elasticity (spring constant α_{tc}) has not been applied to the bead velocity measurement. Therefore, this bead velocity represents a lower bound on the polymerase elongation rate.
16. Interferometer output was low-pass–filtered at 1 kHz and digitized at 1-ms intervals. Before analysis, records were averaged in blocks of 100 points to give a final time resolution of 100 ms. Bead velocity (v_{bead}) was measured as the slope of the line fit to bead position versus time within a 10-s sliding window. Beads achieving $v_{bead} \geq 0.6$ nm s^{-1} continuously for ≥5 s (positive velocity being defined as movement away from the trap center) were judged to be attached to functional transcription complexes and were selected for further analysis. Any interval during which v_{bead} was <0.6 nm s^{-1} continuously for ≥5 s was taken to represent a stall. By this criterion, 13 of 77 complexes spontaneously underwent brief transient stalls that were apparently uncorrelated with the amount of force and were excluded from subsequent analyses. Such events may correspond to transcriptional pausing (*1*, *2*) because movement resumed without the trap being repositioned.
17. Initially, 87 elongating complexes were observed at high laser powers. However, in 10 of them the bead spontaneously detached from its tether before the trap could be repositioned. The site of breakage could not be determined. Data from such complexes were excluded from further analysis. Eleven of the remaining 77 complexes did not stall before reaching the limit of the usable range of the trap.
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19. Although inactive enzyme species induced by chemical stalling in solution can be reactivated by GreB protein [S. Borukhov, V. Sagitov, A. Goldfarb, *Cell* **72**, 459 (1993)], the fraction of complexes that were irreversibly stalled by mechanical load did not decrease when 100 nM GreB protein was included in the buffer (*24*), which suggests either that these complexes are in a different state than that produced in the chemical stalling experiments or that GreB cannot act on complexes adsorbed to cover slips.
20. It is unlikely that the reversible stalling events are due to radiation-induced damage to RNA polymerase, because enzyme photodamage is generally irreversible. The view that reversible stalls are due to applied force, not laser irradiation alone, is further supported by the observation that 95% of reversibly stalled complexes resumed bead movement shortly after the trap was repositioned. Repositioning the trap decreases F_{trap} but increases the laser intensity at the RNA polymerase because the trap is moved closer to the enzyme molecule. Control experiments [at laser powers 82 to 99 mW; all reported laser powers are estimates in the specimen plane determined by the method of (*11*)] in which the trap was repositioned before the stall (in 14 of 15 complexes) subjected the transcription complexes to higher average light intensities and lower average trap forces than those in the stalling experiments. In the controls, complexes were inactivated in 82 ± 58 s (mean ± SD, $n = 15$) exposure to the laser, whereas in the stalling experiments first stalls (reversible or irreversible) occurred significantly earlier, in 38 ± 16 s (mean ± SD, $n = 66$), confirming that the first stalls cannot be explained by photodamage alone.
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22. Stall force measurements are subject to systematic errors, which we attempted to estimate. The two primary sources of error have opposing effects and arise from the fact that the bead motion is not strictly parallel to the cover glass surface but has a vertical component. During elongation, beads experience a force directed downward at an angle θ, where θ is the angle between the DNA and the cover glass (Fig. 1C). This angle varies continuously during the course of an experiment as the DNA tether shortens. Neglect of the downward force component will cause underestimation of the actual force produced by the polymerase by a factor (cosθ). The minimal DNA tether length at stall was estimated by video analysis of the bead Brownian motion (*7*) with the trap shuttered. The tether length was >1915 bp (651 nm) in all experiments, corresponding to θ < 41°. This geometrical consideration alone would lead to a value for F_{stall} that underestimates the applied force by a variable amount up to ~24%. A second source of systematic error, which has the opposite sign, arises from the fact that the interferometer is most sensitive to motion in the plane of the laser beam waist and along the Wollaston shear direction. Polystyrene spheres are initially trapped at a point slightly above the position of the true beam waist (Fig. 1B), and the downward force component acting along the DNA has the effect of carrying the bead into the region of greater detector sensitivity (in contrast, viscous drag calibration experiments, in which purely horizontal forces are generated, are not subject to this artifact). This leads to an overestimate of the distance moved, hence to an overestimate of the force F_{stall}. We estimated the magnitude of this effect at 26% or less, based on experiments in which beads fixed to a cover glass were moved in the specimen plane through the detector at various heights relative to the laser beam waist (*24*). Yet other uncertainties arise because the trap stiffness, hence the restoring force, varies with height, the trap being weaker in the vertical direction than in the horizontal direction. Given these various

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29. Underestimation of F_{stall} (*25*) will cause underestimation of the maximum energy conversion efficiency. Moreover, our efficiency calculation assumes that stalling occurs when the free energy available to drive movement in one cycle of the chemical reaction balances the free energy required to translocate 1 bp along the DNA against the applied force. However, stalling could also occur because of structural alterations in the transcription complex (for example, stabilization of a catalytically inactive enzyme conformation by the applied force). In that case, the maximum efficiency calculated from F_{stall} would underestimate the true efficiency. In contrast, enzymatic consumption of NTPs uncoupled to translocation would reduce the energy conversion efficiency below that calculated from F_{stall}. Although little such consumption has been reported for transcription complexes in solution [for example, see M. Chamberlin, R. L. Baldwin, P. Berg, *J. Mol. Biol.* **7**, 334 (1963)], we cannot exclude the possibility that such uncoupled reactions are catalyzed by immobilized enzyme molecules subjected to the high applied forces used in the stalling experiments. Estimates of energy conversion efficiency from the stall forces of single kinesin and myosin molecules carry analogous uncertainties. Recent evidence (*11*) suggests that kinesin is not tightly coupled near stall.
30. *Escherichia coli* plasmids that carry multiple transcription units can accumulate high densities of transcription-induced supercoils (*31*). This effect can be observed in the absence of DNA gyrase or topoisomerase I activities (which relax negative or positive supercoils, respectively) when one or more of the transcribed genes encodes a membrane-interacting protein (*5*). Specific linking differences (σ) of −0.013 in topoisomerase I mutants [D. N. Cook, D. Ma, N. G. Pon, J. E. Hearst, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10603 (1992)] and 0.024 in the presence of a DNA gyrase inhibitor [H. Y. Wu, S. H. Shyy, J. C. Wang, L. F. Liu, *Cell* **53**, 433 (1988)] have been reported. RNA polymerase transcription is accompanied by an obligatory rotation relative to the DNA helix. Therefore, the supercoiling torque τ_s [($|\sigma|$) 1.4×10^{-19} N m rad^{-1} (*31*)] is expected to exert a force opposing translocation that is estimated by $F_s = 2\pi\tau_s/h$, where h is the pitch of the DNA helix, 3.6 nm. F_s = 3 and 6 pN, respectively, in the two cited cases. However, this calculation may underestimate the force by a factor of ~2 because supercoils in vivo are thought to be confined to a limited portion of the plasmid DNA.
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Minimization of a Polypeptide Hormone

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A stepwise approach for reducing the size of a polypeptide hormone, atrial natriuretic peptide (ANP), from 28 residues to 15 while retaining high biopotency is described. Systematic structural and functional analysis identified a discontinuous functional epitope for receptor binding and activation, most of which was placed onto a smaller ring (Cys^6 to Cys^{17}) that was created by repositioning the ANP native disulfide bond (Cys^7 to Cys^{23}). High affinity was subsequently restored by optimizing the remaining noncritical residues by means of phage display. Residues that flanked the mini-ring structure were then deleted in stages, and affinity losses were rectified by additional phage-sorting experiments. Thus, structural and functional data on hormones, coupled with phage display methods, can be used to shrink the hormones to moieties more amenable to small-molecule design.

The generation of leads for drug design is usually achieved through a laborious discovery process wherein a large number of small molecules are screened for binding to a particular receptor or for modulation of a particular biological response. Although structure-based approaches have been used in some cases for generating candidate molecules directly (*1*), this approach has been limited to binding sites for small substrate molecules or short, continuous peptide segments.

Protein-protein interactions are crucial events in most biological processes and are therefore important targets for drug design. Such interfaces are generally large (600 to more than 1300 Å^2), with 10 to 30 contact side chains on each side of the interface (*2*). Moreover, each patch of contact residues is presented from peptide segments that are often distant in primary sequence. Mimicking such large and discontinuous binding surfaces with rationally designed small molecules is a daunting prospect, but it may be simplified because only a small subset of contact side chains appears to be necessary for tight binding at these interfaces (*3*). Displaying these functional epitopes on minimal structured scaffolds may permit smaller candidate compounds to be generated that bind at protein-protein interfaces.

Atrial natriuretic peptide is a 28-residue peptide hormone that is important for regulation of blood pressure and salt balance (*4*). Smaller ANP peptides produced by the screening of synthetic analogs are at least 500 times weaker in receptor-binding affinity (*5*). It is likely that the binding of ANP to its signaling receptor is highly sensitive to the conformation of ANP, as indicated by the loss of binding caused by the reduction of its single disulfide bond. Using a constrained scaffold designed to preserve the structural presentation of the critical binding determinants, we present a systematic strategy for reducing the size of ANP while maintaining high binding affinity and biopotency.

The first step in our minimization process (Fig. 1) was to determine which ANP residues are important for binding to the extracellular domain of the natriuretic peptide receptor–A (NPR-A) by alanine-scanning mutagenesis (*6*). Each of the 28 residues in ANP was converted to alanine, except those comprising the Cys^7 to Cys^{23} disulfide and Ala^{17}. An enzyme-linked immunosorbent assay (ELISA) of these ANP mutants, produced as peptide fusions with gene III coat protein on phage (*7*), allowed us to rapidly assess the relative binding affinity of the mutants by eliminating the need to purify each peptide to homogeneity. Mutations at only seven positions (Phe^8, Met^{12}, Asp^{13}, Arg^{14}, Ile^{15}, Leu^{21}, and Arg^{27}) each resulted in an affinity that was more than 10 times lower than that of wild-type ANP (Fig. 2). These results are consistent with effects on an aortic ring contraction assay reported for substitutions with D-amino acids or alanines in ANP (*8*).

Five of the seven most important residues (Phe^8, Met^{12}, Asp^{13}, Arg^{14}, and Ile^{15}) formed a small functional epitope on one side of the hormone (Fig. 3A). On the basis of the structure of ANP (*9*), three alternative disulfide forms of the molecule (Cys^7 to Cys^{17}, Cys^6 to Cys^{17}, and Cys^5 to Cys^{17}) were designed to isolate this functional epitope on a smaller disulfide ring and eliminate the native Cys^7 to Cys^{23} disulfide (Fig. 3B). Of these three variants, Cys^6 to Cys^{17} was the best, albeit with an affinity that was more than 100 times lower than

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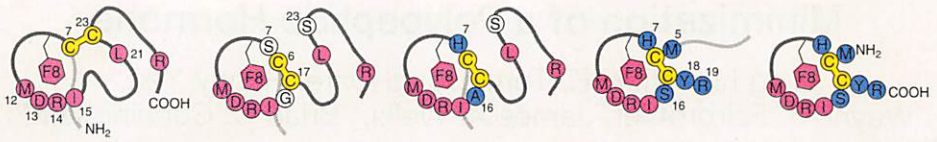
Step 1
Identify binding determinants by alanine scan.
Step 2
Design smaller ring to display primary determinants.
Step 3
Optimize affinity by phage display.
Step 4
Remove residues 20 to 28 and reoptimize.
Step 5
Remove residues 1 to 4 to make 15-residue mini-ANP.

Fig. 1. Strategy used to shrink ANP (28 aa) to mini-ANP (15 aa). **Step 1**: Alanine-scanning mutagenesis of ANP displayed on phage identified seven residues (highlighted in magenta) that were most important for binding to the NPR-A receptor; five of these residues form a tight cluster (Fig. 2). **Step 2**: Based on the structure of ANP (Fig. 3), an alternate Cys^6-Cys^{17} disulfide cross-link (yellow) was designed that reduced binding affinity to a value 100 times lower than that of the wild-type ANP. **Step 3**: Binding affinity was restored by randomly mutating noncritical residues and selecting mutations by phage display that improved affinity (highlighted in blue). **Step 4**: COOH-terminal residues were deleted and the peptide was reoptimized by phage display to yield a 19-aa peptide whose affinity for NPR-A was about 20 times less (weaker) than that of wild-type ANP. **Step 5**: The four NH_2-terminal residues were deleted to yield a 15-aa peptide whose affinity for NPR-A was about five times weaker than that of wild-type ANP.

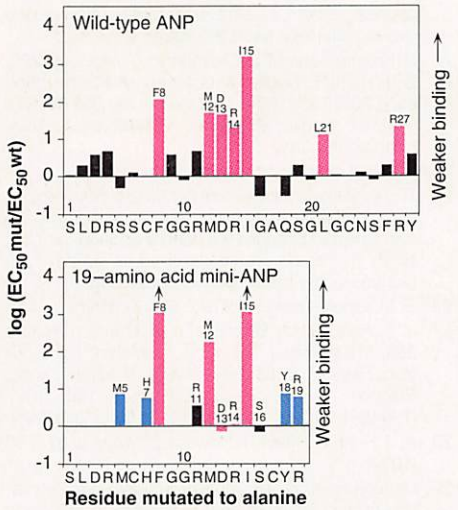

Fig. 2. Functional importance of side chains in wild-type ANP (upper panel) or the optimized 19-aa peptide (lower panel) as assessed by alanine-scanning mutagenesis of the peptides displayed on phage. Binding to the NPR-A receptor was assayed by phage ELISA (*7*) and used to calculate relative affinities expressed as log(EC_{50} mutant/EC_{50} wild type) on the *y* axis. Thus, numbers above zero indicate the reduction in affinity that occurs after side chain atoms beyond the β-carbon (for nonglycine substitutions) are removed by alanine substitution of the wild-type residue shown on the *x* axis. Alanine substitutions in wild-type ANP or the optimized 19-aa peptide, which cause at least 10 times lower affinities, are shown in magenta. Residues that were fixed by phage selection in the optimized 19-aa peptide are shown in blue. Alanine substitutions of Phe^8 and Ile^{15} in the 19-aa peptide result in greater than 1000 times lower affinites (indicated by arrows in lower panel).

Table 1. Relative affinities for the NPR-A receptor of selected ANP variants that were fused to phage or assayed as free peptides (*18*). The affinity for wild-type ANP on phage or as a free peptide was 500 pM and 50 pM, respectively. Numbers in each column indicate the relative reductions in binding compared to wild-type ANP, calculated as EC_{50} mutant/EC_{50} wild type. Phage displaying variants 1 to 5 used a Glu-Gly-Gly spacer fused to gene III at residue + 198; phage displaying variants 6 to 7 used a Gly-Gly-Gly spacer fused at residue +198; phage displaying variants 8 and 9 used a longer $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ spacer fused to gene III at residue +317 (Table 2). Numerical subscripts indicate the position of the preceding cysteines relative to Phe or Cha (cyclohexylalanine) at position 8. Phage affinities were determined by phage ELISA and peptide affinities were measured by RIA (*7*). ND, not determined.

	ANP variant	Relative phage affinity for NPR-A	Relative peptide affinity for NPR-A
1	$SLRRSSC_7FGGRMDRIGAQSGLGC_{23}NSFRY$	1	1
2	$SLRRSSC_7FGGRMDRIGC_{17}QSGLGSNSFRY$	>1,500	ND
3	$SLRRSC_6SFGGRMDRIGC_{17}QSGLGSNSFRY$	104	ND
4	$SLRRC_5SSFGGRMDRIGC_{17}QSGLGSNSFRY$	480	ND
5	$SLRRSC_6HFGGRMDRIAC_{17}QSGLGSNSFRY$	1	ND
6	$SLRRSC_6HFGGRMDRIAC_{17}$	300	40,000
7	$SLRRSC_6HFGGRMDRIAC_{17}$NR-short spacer	8	43
8	$SLRRSC_6HFGGRMDRIAC_{17}$NR-long spacer	50	43
9	$SLRRMC_6HFGGRMDRISC_{17}$YR-long spacer	8	6
10	$MC_6HFGGRMDRISC_{17}YR$	ND	7
11	RC_7(Cha)$GGRIDRIFRC_{18}$	ND	7,200

that of wild-type ANP (Table 1, variants 2 to 4). Despite this large reduction in binding, the Cys^6 to Cys^{17} variant binds NPR-A more tightly than reported for the Cys^7 to Cys^{18} or linear ANP forms (*10*, *11*).

Monovalent phage display (*12*) was applied to improve the affinity of the Cys^6 to Cys^{17} ANP variant (Fig. 1, step 3). Noncritical residues (Ser^7, Gly^9, Gly^{10}, Arg^{11}, and Gly^{16}) within the Cys^6 to Cys^{17} disulfide loop were mutated simultaneously to all 20 possibilities (Table 2, library 1). After six rounds of selecting phage for binding NPR-A immobilized on plates, virtually every phage selected contained S7H and G16A (*13*), whereas the wild-type residues Gly^9, Gly^{10}, and Arg^{11} were retained (*14*). This mutant had nearly the same affinity as wild-type ANP and therefore was 100 times improved compared with the starting Cys^6 to Cys^{17} mutant (Table 1, variant 5).

We next shortened the COOH-terminal portion of the molecule by deleting residues Gln^{18} through Tyr^{28} (Fig. 1, step 4). This reduced the affinity by 300 times presumably as a result of the loss of some binding determinants (Leu^{21} and Arg^{27}) and a possible distortion of the primary binding epitope (Table 1, variant 6). To recover binding affinity, we sorted three new phage display libraries (Table 2, libraries 2 to 4). These contained two, three, or four randomized residues after Cys^{17}. Most of the phage selected from these libraries incorporated Q18N and S19R.

The 19–amino acid (aa) peptide containing the Q18N and S19R substitutions bound to NPR-A with an affinity that was only eight times weaker than that of the wild-type ANP when displayed on phage (Table 1, variant 7). This peptide was synthesized, and the binding affinity to NPR-A was determined to be 43 times weaker than that of the wild-type ANP. We also synthesized the prototype 17-aa peptide (Table 1, variant 6); instead of its binding being 300 times weaker than that of wild type (as expected from the phage ELISA), it was 40,000 times weaker. We hypothesized that this discrepancy (a factor of 5 to 100) between the binding of peptides fused to phage compared with the free peptide could result if the sequence of the gene III protein near the cyclic peptide contributed fortuitous binding determinants. To test this, we placed a longer spacer sequence $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ after residue 19 and fused it to a different place in the gene III protein (beginning at position 317). Indeed, the binding of this analog was 50 times weaker than that of the wild-type ANP, a value consistent with the 43 times lower binding measured for the synthetic peptide (Table 1, variant 8).

The 19-aa peptide was then reoptimized on phage with the use of the longer spacer, by randomizing the four residues immediately flanking the Cys^6 to Cys^{17} disulfide bond (Table 2, library 5). The selectant with highest affinity introduced S5M, A16S, and N18Y and retained His^7. On phage this variant bound only eight times weaker than wild-type ANP and as an isolated peptide only six times weaker (Table 1, variant 9).

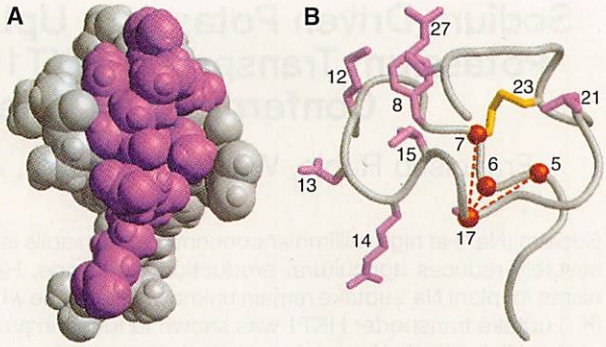

Fig. 3. Structural models of ANP used to design smaller versions of the hormone. (**A**) Space-filling model showing the clustering of NPR-A receptor-binding determinants identified by alanine scanning (Fig. 2). The binding determinants Phe[8], Met[12], Asp[13], Arg[14], Ile[15], Leu[21], and Arg[27] (highlighted in magenta) account for most of the hormone's receptor-binding affinity. The model was derived from nuclear magnetic resonance structural coordinates of an ANP variant (*20*). (**B**) α-Carbon tracing illustrating three proposed alternate disulfide cross-links (red) that places much of the functional epitope on smaller rings. The ensemble average distances, between the α-carbons at position 17 and the proposed cross-links to positions 5, 6, and 7, for 11 energy-minimized structures, are 6.6 ± 1.2, 7.3 ± 1.2, and 8.8 ± 0.6 Å, respectively. The native 7 to 23 disulfide bond and the seven most dominant binding determinants are shown in yellow and magenta, respectively. The image in (B) is rotated left 90° about its vertical axis relative to the image in (A).

Table 2. Phage libraries displaying randomized ANP molecules. Residues designated as *X* were randomized to all 20 natural amino acids as described (*19*). Each library consisted of 4×10^7 to 22×10^7 independent transformants and theoretically covered all possible sequence combinations. The randomized ANP molecules were fused via a glycine-rich spacer to the COOH-terminal gene III segment as shown below.

	Positions randomized	Spacer	Position of gene III fusion
1	SLDRSC$_6$XFXXX-MDRIXC$_{17}$QSGL-GSNSFRY	EGG	+198
2	SLDRSC$_6$HFG-GRMDRIAC$_{17}$XX	GGG	+198
3	SLDRSC$_6$HFGG-RMDRIAC$_{17}$XXX	GGG	+198
4	SLDRSC$_6$HFG-GRMDRIAC$_{17}$XXXX	GGG	+198
5	SLDRXC$_6$XFGG-RMDRIXC$_{17}$XR	(GGGS)$_2$	+317

We analyzed the functional importance of the selected and conserved residues in the optimized 19-aa peptide by alanine scanning and phage ELISA (Fig. 2B). The most important determinants were the hydrophobic side chains Phe[8], Met[12], and Ile[15], which decreased the affinity 100 to 1000 times when converted to alanine. These were also the most dominant determinants in the full-length ANP (Fig. 2A), suggesting that the basic structural and functional epitopes were similar whether presented from the wild-type scaffold or the shortened peptide. These residues appear to be more important in the context of the smaller scaffold, indicating that the functional epitope may be more concentrated. However, three of the important residues in the wild-type hormone (Asp[13], Arg[14], and Leu[21]) were no longer important in the selected 19-aa peptide. Perhaps their roles had been replaced by the newly selected residues, Met[5], His[7], Tyr[18], and Arg[19]. Indeed, when any one of these was converted to alanine, the affinity decreased 8 to 10 times (Fig. 2B). Thus, this minimization process appeared to preserve the dominant features of the functional epitope while recruiting local functional determinants. These changes compensate for the perturbations caused by the deletions and the alternative Cys[6] to Cys[17] disulfide bond.

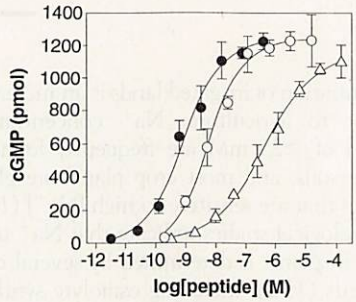

Fig. 4. Potency of mini-ANP peptides for stimulating cGMP production in 293 cells transiently expressing NPR-A (*21*). EC_{50} values calculated from four-parameter curve fits were 0.78 nM for wild-type ANP (●), 5.9 nM for variant 10 (○, mini-ANP), and 430 nM for variant 7 (△). Each data point and error bar represents the mean of three replicates ± SD.

Finally, we deleted the first four residues on the optimized 19-aa peptide (Fig. 1, step 5) because the alanine scan of the wild-type ANP showed that these residues were only of minor importance. The resulting 15-aa peptide was synthesized and then amidated on its COOH-terminus to better mimic the presentation of the peptide on phage. This peptide, which we call mini-ANP, bound with an affinity that was only seven times lower than that of the wild-type ANP (Table 1, variant 10). We measured the biopotency of several of these peptides (Fig. 4) by their ability to stimulate guanosine 3′5′-monophosphate (cGMP) production in cells that express NPR-A (*14*). The median effective concentration (EC_{50}) for wild-type ANP was 0.8 nM compared with 5.9 nM for the mini-ANP 15-aa peptide (variant 10) and 430 nM for the 19-aa peptide (variant 7). This biopotency corresponds to a 7.6 times lower affinity for the mini-ANP variant relative to wild-type ANP and is consistent with the 7.4 times lower affinity for NPR-A binding measured for this peptide (*15*). Thus, the size of the hormone was reduced nearly 50% while retaining high binding affinity for NPR-A (EC_{50} = 0.48 nM) and biopotency (EC_{50} = 5.9 nM). This can be compared with one of the best reported smaller versions of ANP, a 13-aa peptide derived by more standard medicinal chemistry methods (*11*) with an EC_{50} of 470 nM or 7200 times lower affinity relative to the wild-type ANP (Table 1, variant 11).

Previous studies have shown that it is possible to graft protein-binding determinants between antibodies or structurally homologous hormone scaffolds (*16*). In contrast, attempts to place catalytic groups from a serine protease onto a cyclic peptide without preserving the original structure have failed (*17*). Our structure-based approach to minimization attempts to preserve the functional epitope while maintaining its structure on a smaller scaffold. Phage display was paramount for rectifying the imperfections present in the smaller designs. This method may be generally useful for generating peptide leads for other polypeptide hormones.

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7. B. C. Cunningham, D. G. Lowe, B. Li, B. D. Bennett, J. A. Wells, *EMBO J.* **13**, 2508 (1994). A phage ELISA was used to quantitate the binding affinity of ANP displaying phage to NPR-A. Alanine mutants of ANP were displayed on the pB1537 phagemid that contained two additional mutations (R3D in ANP and Q29E in the spacer residue immediately after residue 28 of ANP) that increase the efficiency of ANP display without affecting NPR-A–binding affinity (B. Li *et al.*, unpublished data). For Fig. 2 (upper panel) affinities were based on phage ELISA measurements, except those for F8A and I15A, which were determined from radioimmunoassay (RIA) measurements of the respective peptides.
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18. Peptides were synthesized on an Applied Biosystems 430A automated synthesizer according to standard *N-tert*-butoxycarbonyl chemistry protocols, cleaved off the resin with anhydrous HF at 0°C, then cyclized by the potassium ferricyanide method and purified by reversed-phase high-performance liquid chromatography. Masses of each peptide were verified by mass spectrometry. All the peptides except for variant 6 were synthesized with an amidated COOH-terminus.
19. Phagemid libraries that displayed variants of ANP contained codons that were randomized to all 20 amino acids by site-directed mutagenesis [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)] with the following oligonucleotides that changed target codons to NNS sequences (N is a mixture of all four bases and S is a mixture of G and C bases): 5′-TCTCTGGATAGATCTTGC(NNS)TTC(NNS)$_3$ ATGGATCGTATC(NNS)TGCCAGAGCGGT-3′ for library 1, 5′-ATGGATCGTATCGCGTGC(NNS)$_2$·GGGGGCGGCCC-3′ for library 2, 5′-ATGGATCGTATCGCGTGC(NNS)$_3$·GGGGGCGGG-CCC-3′ for library 3, 5′-ATGGATCGTATCGCTGC(NNS)$_4$·GGGG-GCGGGCCC-3′ for library 4, and 5′-TATGCATCTCTGGATAGA(NNS)TGC(NNS)TTCGGGGGCCGGATGGATCGTATC(NNS)TGC(NNS)CGGGGGGGCGGGTCT-3′ for library 5. Each library consisted of 4×10^7 to 22×10^7 independent mutants and theoretically represented all possible sequence combinations. To minimize wild-type background, the mutagenesis template used for libraries 2 to 5 was frameshifted within the region to be randomized.
20. The molecular models (Fig. 3) are from 1 of 11 energy-minimized structures of an ANP variant (*9*) whose coordinates are deposited in the Brookhaven Protein Data Bank (accession number 1ANP). This model of wild-type ANP contains two substitutions (L12M-S14R), which revert back mutations that were present in the variant whose structure was solved.
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22. We thank D. Lowe for providing the immunoglobulin G–receptor fusion protein and for advice; B. McDowell for discussions; the DNA synthesis group for oligonucleotides; A. Padua for amino acid analysis; and D. Woods, K. Andow, and W. Anstine for help with graphics.

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Sodium-Driven Potassium Uptake by the Plant Potassium Transporter HKT1 and Mutations Conferring Salt Tolerance

Francisco Rubio, Walter Gassmann, Julian I. Schroeder

Sodium (Na^+) at high millimolar concentrations in soils is toxic to most higher plants and severely reduces agricultural production worldwide. However, the molecular mechanisms for plant Na^+ uptake remain unknown. Here, the wheat root high-affinity potassium (K^+) uptake transporter HKT1 was shown to function as a high-affinity K^+-Na^+ cotransporter. High-affinity K^+ uptake was activated by micromolar Na^+ concentrations; moreover, high-affinity Na^+ uptake was activated by K^+ (half-activation constant, 2.8 μM K^+). However, at physiologically detrimental concentrations of Na^+, K^+ accumulation mediated by HKT1 was blocked and low-affinity Na^+ uptake occurred (Michaelis constant, ~16 mM Na^+), which correlated to Na^+ toxicity in plants. Point mutations in the sixth putative transmembrane domain of HKT1 that increase Na^+ tolerance were isolated with the use of yeast as a screening system. Na^+ uptake and Na^+ inhibition of K^+ accumulation indicate a possible role for HKT1 in physiological Na^+ toxicity in plants.

Salinization of irrigated lands is an increasing threat to agriculture. Na^+ concentrations [Na^+] of ≥25 mM are frequently found in saline soils, and most crop plants are glycophytes that are sensitive to high [Na^+] (*1*, *2*). Physiological studies indicate that Na^+ tolerance in plants is determined by several components (*1*, *2*), including osmolyte synthesis (*3*), Na^+ sensitivity of vital enzymes (*4*), and ion transport processes (*5*–*9*). It has been suggested that reducing Na^+ accumulation is crucial for engineering Na^+ tolerance in plants (*8*, *10*). However, the molecular mechanisms for Na^+ uptake and exclusion in plants remain unknown.

Recently, a complementary DNA (cDNA) from wheat, *HKT1*, was isolated that encodes a high-affinity K^+ uptake transporter (*11*), and in roots of *Arabidopsis thaliana*, voltage-clamp recordings of high-affinity K^+ uptake were reported (*12*). Current-voltage studies of the mechanism of K^+ uptake through HKT1 showed a variation in HKT1-mediated currents, which indicated that additional relevant transported factors remained unknown (*11*). Initial experiments showed that HKT1-mediated currents are affected by Na^+ (*11*). Here, we show that HKT1 mediates high-affinity K^+-Na^+ cotransport and low-affinity Na^+ uptake in a manner consistent with Na^+ toxicity in plants, and we identify mutations that improve HKT1-mediated Na^+ tolerance.

In control tracer flux experiments (*13*) with untransformed K^+ uptake–deficient yeast strains (*14*), neither $^{86}Rb^+$ (K^+) nor $^{22}Na^+$ uptake was observed (Fig. 1, filled symbols). In HKT1-expressing yeast cells, Na^+ strongly stimulated Rb^+ uptake (half-activation constant $K_{1/2}$ = 175 ± 50 μM Na^+, mean ± SD; n = 4) (Fig. 1A). $^{22}Na^+$ flux experiments in yeast allowed us to determine whether Na^+ uptake was mediated by HKT1 or whether Na^+ was merely enhancing Rb^+ accumulation (*15*). $^{22}Na^+$ uptake experiments in HKT1-expressing yeast demonstrat-

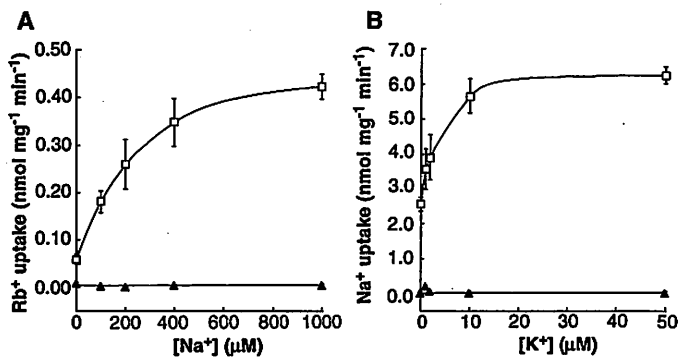

Fig. 1. HKT1 expressed in yeast gives rise to Na^+-stimulated Rb^+ uptake (**A**) and to K^+-stimulated Na^+ uptake (**B**) [□, yeast cells expressing HKT1; ▲, K^+ uptake–deficient yeast mutants (*14*)]. In (A), the initial rate of Rb^+ uptake is represented as a function of increasing external Na^+. In (B), the initial rate of Na^+ uptake is represented as a function of increasing external K^+. Uptake experiments were carried out at pH 6.0, 30°C, in the presence of 15 μM Rb^+ (A) or 200 μM Na^+ (B) (*13*). At nominally 0 [Na^+], a Rb^+ uptake rate of 0.05 nmol mg^{-1} min^{-1} was observed (A), and at nominally 0 [K^+], a Na^+ uptake rate of 2.6 nmol mg^{-1} min^{-1} was observed (B). This can likely be attributed to the background [Na^+] (11 μM) and [K^+] (2 μM) in the buffer used for the uptake experiments, as determined by atomic absorption spectrophotometry.

ed that Na^+ uptake was mediated by HKT1 (Fig. 1B). Moreover, K^+ stimulated the uptake of Na^+ ($K_{1/2}$ = 2.8 ± 0.3 μM K^+; n = 3) (Fig. 1B). HKT1-mediated Rb^+ uptake was not affected by external pH (*16*).

Cotransport of K^+ and Na^+ was directly quantified by studies of HKT1-mediated currents in *Xenopus* oocytes (*17*). Extracellular simultaneous perfusion with 1 mM Na^+ and 0.3 mM K^+ produced downward deflections in the current trace that were larger than those produced with 1 mM Na^+ or 0.3 mM K^+ alone (n = 21) (Fig. 2A). Absolute current values showed that Na^+ or K^+ alone merely reduced positive (outward) current (Fig. 2A) (*11*). However, the combined addition of Na^+ and K^+ produced inward currents and cation uptake (Fig. 2A). Additional experiments suggested that among the alkali metal cations (Li^+, Na^+, K^+, Rb^+, and Cs^+), Na^+ was at least 10 times as effective as any of the other cations in activating K^+ uptake (n = 5 for each cation) (*18*). Uninjected oocytes did not show currents under the same conditions (n = 33) (Fig. 2B).

In contrast to Na^+-free conditions (*11*), the simultaneous exposure of HKT1-expressing oocytes to Na^+ and K^+ produced stable reversal potentials (n = 55). Therefore, the identity of the ions transported by HKT1 in oocytes could be unequivocally established by determining absolute shifts in reversal potentials of HKT1-mediated steady-state currents under varying external [Na^+] or [K^+] (Fig. 2, C and D). HKT1-mediated steady-state currents in oocytes were insensitive to external pH in the range of pH 5 to 8 (n = 11) (*16*). Increasing external [Na^+] from 0.3 to 10 mM, with [K^+] constant at 1 mM, produced shifts of current-voltage curves toward more positive potentials (Fig. 2C), which confirmed that Na^+ was taken up by HKT1. At 1 mM external [Na^+], increasing [K^+] from 0.3 to 10 mM caused shifts in reversal potentials that were more pronounced (Fig. 2D). An increase in [K^+] from 1 to 10 mM caused a shift of +38 ± 6 mV (n = 8), whereas an increase in [Na^+] from 1 to 10 mM caused a shift of +22 ± 4 mV (n = 9). This result would be consistent with a transport stoichiometry of 1.7 K^+ to 1 Na^+ (*19*). Uptake experiments in yeast with micromolar concentrations of K^+ ($^{42}K^+$) and Na^+ ($^{22}Na^+$) showed a stoichiometry of 2.1 ± 0.5 to 1 (n = 8), which is in agreement with results obtained in oocyte experiments.

We next investigated the effect of phytotoxic high millimolar concentrations of Na^+ (>25 mM) on HKT1-mediated K^+ uptake in yeast. At a physiological [K^+] (100 μM), high [Na^+] inhibited the growth of HKT1-expressing yeast cells (Fig. 3A, left). This finding enabled us to design a strategy for isolating HKT1 mutants, using yeast as a screening system to identify domains in HKT1 responsible for Na^+ toxicity. HKT1 mutants were isolated (*20*) that allowed yeast cells to grow in the presence of 300 mM Na^+ and 0.1 mM K^+ (Fig. 3A, center and right). Two strongly Na^+-tolerant mutations were obtained, $Ala^{240} \rightarrow Val^{240}$ and $Leu^{247} \rightarrow Phe^{247}$, that were both located in the sixth hydrophobic domain of HKT1. The growth rates of yeast cells expressing the two mutated versions and the wild-type HKT1 showed no differences in the presence of nontoxic micromolar concentrations of Na^+. In addition, at low micromolar cation concentrations, the two mutants and the wild type showed similar rates of high-affinity Rb^+ and Na^+ uptake (n = 30) (*21*). However, at 300 mM Na^+ and 0.1 mM K^+, yeast cells expressing the A240V and L247F mutations (*22*) showed growth rates far in excess of that of the strain expressing wild-type HKT1 (Fig. 3B, inset).

Tracer flux experiments in yeast showed inhibition of HKT1-mediated Rb^+ uptake

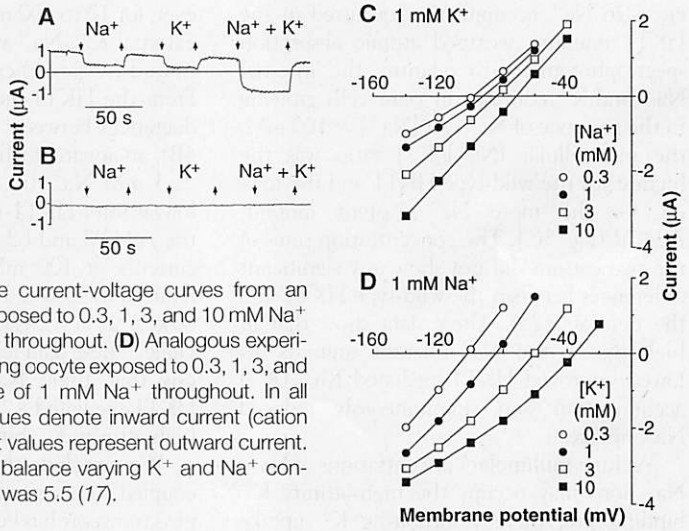

Fig. 2. HKT1 expressed in oocytes gives rise to Na^+-coupled K^+ uptake. (**A**) Exposure of an HKT1-expressing oocyte to 1 mM Na^+, 0.3 mM K^+, and 1 mM Na^+ plus 0.3 mM K^+. (**B**) Uninjected oocyte treated as in (A). The membrane potential in (A) and (B) was held at −120 mV. (**C**) Steady-state current-voltage curves from an HKT1-expressing oocyte exposed to 0.3, 1, 3, and 10 mM Na^+ in the presence of 1 mM K^+ throughout. (**D**) Analogous experiment with an HKT1-expressing oocyte exposed to 0.3, 1, 3, and 10 mM K^+ in the presence of 1 mM Na^+ throughout. In all panels, negative current values denote inward current (cation uptake), and positive current values represent outward current. Tris-glutamate was used to balance varying K^+ and Na^+ concentrations; the external pH was 5.5 (*17*).

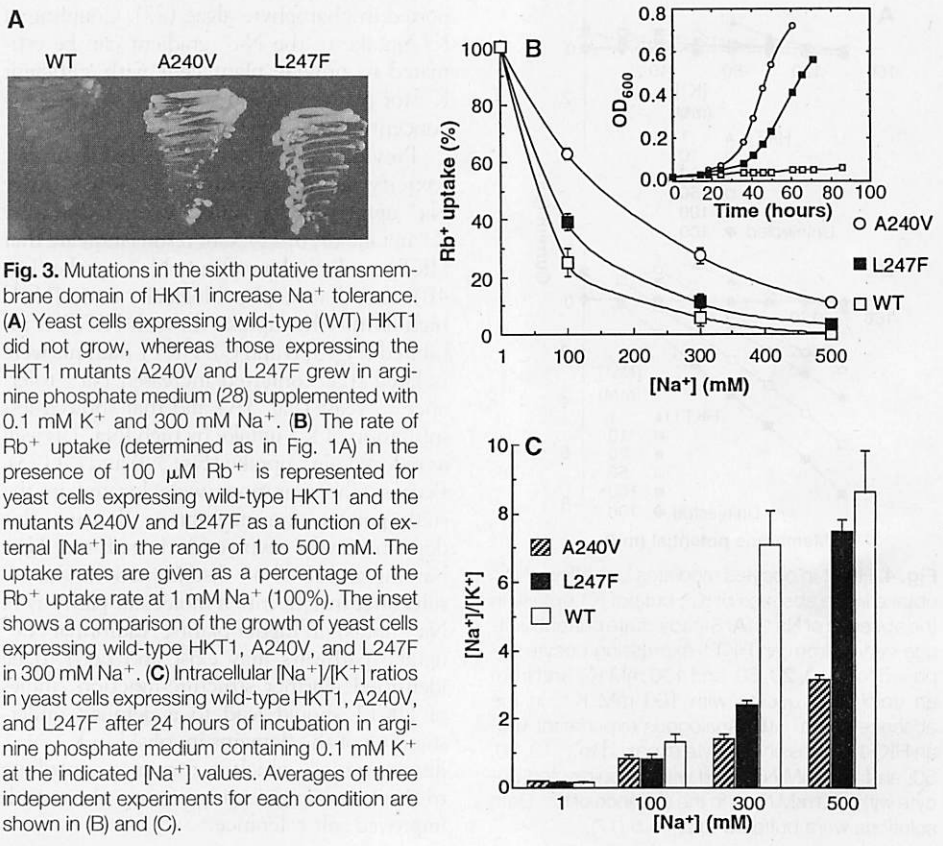

Fig. 3. Mutations in the sixth putative transmembrane domain of HKT1 increase Na^+ tolerance. (**A**) Yeast cells expressing wild-type (WT) HKT1 did not grow, whereas those expressing the HKT1 mutants A240V and L247F grew in arginine phosphate medium (*28*) supplemented with 0.1 mM K^+ and 300 mM Na^+. (**B**) The rate of Rb^+ uptake (determined as in Fig. 1A) in the presence of 100 μM Rb^+ is represented for yeast cells expressing wild-type HKT1 and the mutants A240V and L247F as a function of external [Na^+] in the range of 1 to 500 mM. The uptake rates are given as a percentage of the Rb^+ uptake rate at 1 mM Na^+ (100%). The inset shows a comparison of the growth of yeast cells expressing wild-type HKT1, A240V, and L247F in 300 mM Na^+. (**C**) Intracellular [Na^+]/[K^+] ratios in yeast cells expressing wild-type HKT1, A240V, and L247F after 24 hours of incubation in arginine phosphate medium containing 0.1 mM K^+ at the indicated [Na^+] values. Averages of three independent experiments for each condition are shown in (B) and (C).

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with increasing $[Na^+]$ in the range of 1 to 500 mM (Fig. 3B, lower trace). Millimolar concentrations of Na^+ inhibit K^+ uptake in plants (*6*, *12*), which could lead to K^+ deficiency. In the Na^+-tolerant HKT1 mutants, the rate of high-affinity Rb^+ uptake was less reduced by high $[Na^+]$ than in the wild type (n = 9) (Fig. 3B). The more Na^+-resistant mutant, A240V, showed less inhibition of Rb^+ uptake (Fig. 3B). The Na^+ concentrations at which Rb^+ uptake was reduced to 50% were 58 ± 5.3 mM Na^+ (wild-type HKT1), 154 ± 6.1 mM Na^+ (A240V), and 77 ± 5 mM Na^+ (L247F) (n = 3).

To determine whether additional differences in Na^+ accumulation occurred in the HKT1 mutants, we used atomic absorption spectrophotometry to quantify the internal Na^+ and K^+ contents in yeast cells growing in the presence of Na^+. At $[Na^+] > 100$ mM, the intracellular $[Na^+]/[K^+]$ ratio was the highest for the wild-type HKT1 and the lowest for the more Na^+-resistant mutant, A240V (Fig. 3C). The concentration sums of the two cations did not show any significant differences between the wild-type HKT1 and the mutants (*23*). These data show that at high $[Na^+]$, the Na^+-tolerant mutants allowed improved HKT1-mediated Rb^+ (K^+) accumulation and simultaneously reduced Na^+ uptake.

At high millimolar concentrations of Na^+, Na^+ ions may occupy the high-affinity K^+ binding site, thereby inhibiting K^+ uptake (Fig. 3B) and allowing increased Na^+ uptake (Fig. 3C) (*23*). To test this hypothesis, we studied currents in oocytes at high millimolar concentrations of Na^+ or K^+. In uninjected control oocytes, $[Na^+]$ or $[K^+]$ in the range of 1 to 100 mM did not produce inward currents (n = 14) (Fig. 4). In HKT1-expressing oocytes, external $[K^+]$ in the range of 1 to 100 mM in the absence of Na^+ reduced outward currents (*11*), but could not stimulate appreciable inward currents; this result underscores the necessity of Na^+ coupling for K^+ uptake (n = 9) (Fig. 4A). Similarly, 100 mM Li^+, Rb^+, or Cs^+ produced inward currents within the resolution range (n = 8) (*24*, *25*). However, for 10 to 100 mM Na^+ in the absence of external K^+, Na^+ was able to generate large inward (negative) currents (n = 9) (Fig. 4B). From the HKT1-mediated inward Na^+ conductances between −120 and −140 mV (Fig. 4B), an apparent Michaelis constant K_m of 16 ± 3 mM Na^+ (n = 5) was determined for low-affinity HKT1-mediated Na^+ uptake. In the A240V and L247F mutants, inward Na^+ currents at 100 mM Na^+ and 0 K^+ were reduced to 33% ± 10% (A240V, n = 17) and 66% ± 22% (L247F, n = 11) of the wild-type value. These data lend support to the hypothesis that toxic Na^+ concentrations block HKT1-mediated K^+ uptake by competing for high-affinity K^+ binding.

We conclude that HKT1 acts as a Na^+-coupled cotransporter, even though Na^+-coupled transport has been classically thought not to occur in higher plants (for reviews, see *1*, *9*, *26*). Na^+-coupled K^+ transport has been reported in charophyte algae (*27*). Coupling of K^+ uptake to the Na^+ gradient can be estimated to provide plant cells with sufficient K^+ for plant nutrition from soil solution K^+ concentrations of ~1 μM (*19*).

Previous studies have suggested that Na^+ toxicity is accompanied by low-affinity Na^+ uptake and by inhibition of high-affinity K^+ uptake (*5*, *6*, *12*). Our results indicate that HKT1 mediates low-affinity Na^+ uptake (Fig. 4B), and that at physiologically toxic $[Na^+]$ high-affinity K^+ uptake through HKT1 is inhibited (Fig. 3, B and C). HKT1 mutants were isolated that conferred increased Na^+ tolerance to yeast (Fig. 3A) and that showed less inhibition of K^+ uptake by high $[Na^+]$ as well as reduced Na^+ uptake (Fig. 3, B and C) (*23*). Because the mutations were located in the sixth putative transmembrane domain, this domain may be involved in low-affinity Na^+ binding and K^+-Na^+ discrimination. Our results offer insight into a molecular pathway of Na^+ uptake in higher plants. Additional Na^+ uptake pathways may exist and need to be identified. Further structure-function studies of HKT1 should lead to a detailed understanding of the domains involved in K^+-Na^+ discrimination, which in turn may contribute to the future engineering of crop plants with improved salt tolerance.

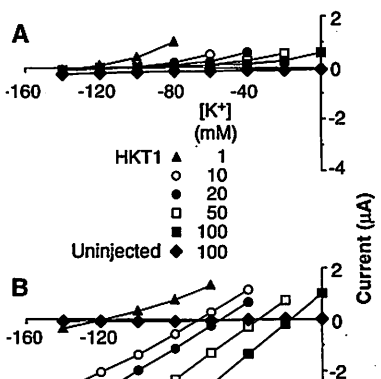

Fig. 4. HKT1 in oocytes mediates low-affinity Na^+ uptake in the absence of K^+, but not K^+ uptake in the absence of Na^+. (**A**) Steady-state current-voltage curves from an HKT1-expressing oocyte exposed to 1, 10, 20, 50, and 100 mM K^+ and from an uninjected oocyte with 100 mM K^+, in the absence of Na^+. (**B**) Analogous experiment with an HKT1-expressing oocyte exposed to 1, 10, 20, 50, and 100 mM Na^+ and with an uninjected oocyte with 100 mM Na^+, in the absence of K^+. Bath solutions were buffered to pH 5.5 (*17*).

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13. The *HKT1* cDNA was subcloned under the control of the yeast *PMA1* gene promoter [R. Serrano, M. C. Kielland-Brandt, G. R. Fink, *Nature* **319**, 689 (1986)] in plasmid pDR195. For uptake experiments, K^+-starved cells [J. Ramos and A. Rodríguez-Navarro, *Eur. J. Biochem.* **154**, 307 (1986)] were analyzed as described (*28*).
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16. Exposure in yeast to pH 8 for durations of >8 min resulted in a decrease of Rb^+ uptake compared to pH 6, leading to an apparent proton stimulation (*11*). However, in experiments in which the initial rate of Rb^+ uptake was determined at pH 6 and 8, no differences in initial uptake rates were observed (n = 3). In oocytes, HKT1-mediated outward currents are evident in the absence of external substrate (see Fig. 2) (*11*). The effects of pH on reversal potentials in oocytes (*11*) were obtained by subtraction of HKT1-mediated pre–steady-state outward currents from each other, which gave rise to apparent pH effects (*11*).
17. Oocytes 1 day after injection with 20 ng of *HKT1* mRNA (*11*) were voltage-clamped while bathed in a solution containing 6 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM Mes-tris (pH 5.5), with osmolality of 240 to 260 mosmol kg^{-1} with D-sorbitol. K^+ and Na^+ were added as glutamate salts.
18. W. Gassmann, F. Rubio, J. I. Schroeder, data not shown.
19. The theoretical reversal potential E_{rev} of a K^+-Na^+ cotransporter is defined by

$$E_{rev} = \frac{58\ mV}{n+m}\left\{m\left[\log\left(\frac{[K^+]_{ext}}{[K^+]_{cyt}}\right)\right] + n\left[\log\left(\frac{[Na^+]_{ext}}{[Na^+]_{cyt}}\right)\right]\right\}$$

where $[K^+]_{ext}$, $[K^+]_{cyt}$, $[Na^+]_{ext}$, and $[Na^+]_{cyt}$ are the external and cytosolic K^+ and Na^+ activities, respectively, and the ratio m:n is the transport stoichiometry. For a stoichiometry of 2 K^+ to 1 Na^+, the shift in reversal potential would be +38.7 mV for an order of magnitude increase in $[K^+]_{ext}$ and +19.3 mV for an order of magnitude increase in $[Na^+]_{ext}$. Assuming physiological values of 0.1 mM for $[Na^+]_{ext}$, 1 mM for $[Na^+]_{cyt}$ (*2*), and 100 mM for $[K^+]_{cyt}$, and a membrane potential of −200 mV (*9*), it can be calculated that a 1 K^+ to 1 Na^+ cotransporter could take up K^+ from soil solutions containing ≥127 nM K^+, and a 2 K^+ to 1 Na^+ cotransporter could take up K^+ from soil solutions containing ≥2.13 μM K^+.
20. A library of 20 × 10^3 randomly mutated HKT1-containing pYES2 plasmids (Invitrogen, San Diego, CA) was generated by treating the plasmid containing *HKT1* cDNA in vitro with 7% hydroxylamine for 2

hours [R. S. Sikorski and J. D. Boeke, in *Guide to Yeast Genetics and Molecular Biology*, C. Guthrie and G. R. Fink, Eds. (Academic Press, San Diego, CA, 1991), vol. 194, pp. 302–318]. Competent cells of K^+ uptake–deficient yeast (*14*) were transformed with the mutagenized HKT1 library. The transformants were eluted and plated in arginine phosphate medium (*28*) containing 0.1 mM K^+ and 500 mM Na^+, which inhibited the growth of the strain expressing wild-type HKT1. Growing colonies were selected, and their plasmids were isolated and reintroduced into yeast to retest for growth in the presence of high [Na^+]. Ten of 60 plasmids conferred the ability to grow at high [Na^+]. The *HKT1* cDNAs of these 10 plasmids were sequenced to identify mutations. The cDNA of the HKT1 mutants was subcloned under the control of the yeast *PMA1* gene promoter, as described (*13*).

21. F. Rubio, W. Gassmann, J. I. Schroeder, data not shown.
22. Abbreviations for the amino acid residues are as follows: A, Ala; F, Phe; L, Leu; and V, Val.
23. For example, at 0.1 mM K^+ and 500 mM Na^+, intracellular K^+ + Na^+ contents were 301 ± 35 nmol mg^{-1} (HKT1), 314 ± 20 nmol mg^{-1} (A240V), and 295 ± 13 nmol mg^{-1} (L247F).
24. W. Gassmann, F. Rubio, J. I. Schroeder, data not shown.
25. Uptake experiments in yeast, in which the background buffer [Na^+] was 11 μM (see Fig. 1), showed that the maximal velocity (V_{max}) for Na^+ uptake at [Na^+] ≥ 1 mM (17 nmol mg^{-1} min^{-1}) was nine times that of the V_{max} for Rb^+ uptake at [Rb^+] ≥ 1 mM (1.8 nmol mg^{-1} min^{-1}).
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29. We thank R. F. Gaber and A. Rodríguez-Navarro for providing yeast strains, W. B. Frommer for providing the plasmid pDR195 and A. Rodríguez-Navarro, N. M. Crawford, A. Schinder, and J. M. Ward for comments. Supported by U.S. Department of Agriculture (95-37304-2227) and Department of Energy (DE-FG03-34-ER20148) grants and a Presidential Young Investigator Award (to J.I.S.) and a Ministerio de Educación y Ciencia (Spain) postdoctoral fellowship (to F.R.).

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A Human Telomeric Protein

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Telomeres are multifunctional elements that shield chromosome ends from degradation and end-to-end fusions, prevent activation of DNA damage checkpoints, and modulate the maintenance of telomeric DNA by telomerase. A major protein component of human telomeres has been identified and cloned. This factor, TRF, contains one Myb-type DNA-binding repeat and an amino-terminal acidic domain. Immunofluorescent labeling shows that TRF specifically colocalizes with telomeric DNA in human interphase cells and is located at chromosome ends during metaphase. The presence of TRF along the telomeric TTAGGG repeat array demonstrates that human telomeres form a specialized nucleoprotein complex.

Human chromosomes carry a long terminal array of double-stranded TTAGGG hexamers that are maintained by telomerase. Telomeric DNA is thought to form a protective nucleoprotein cap through its association with telomere-specific proteins (*1*). Because the loss of telomere function can induce cell cycle arrest and genome instability, the telomeric complex is likely to be required in all human cells. Changes in the structure and function of human telomeres are thought to play a role in malignant transformation and cellular senescence (*2*, *3*).

Protein components of the telomeric complex have been identified in ciliates and in yeast, but not in vertebrate systems (*1*). Quests for vertebrate telomeric proteins had previously yielded a single candidate factor that could potentially bind along the length of the telomeric TTAGGG repeat array (*4*–*6*). This protein, TRF (telomeric repeat binding factor), associates with double-stranded TTAGGG repeat arrays in vitro and displays strong specificity for vertebrate telomeric DNA (*4*, *5*). TRF does not bind to single-stranded telomeric sequences and does not require the proximity of a DNA terminus for its interaction (*4*). The activity is expressed in nuclei from human, monkey, rodent, and chicken cells, which all carry TTAGGG repeat arrays at their chromosome ends (*4*). Here, we show that TRF is a protein component of human telomeres.

Human TRF (hTRF) activity can be detected in HeLa cell nuclear extracts on

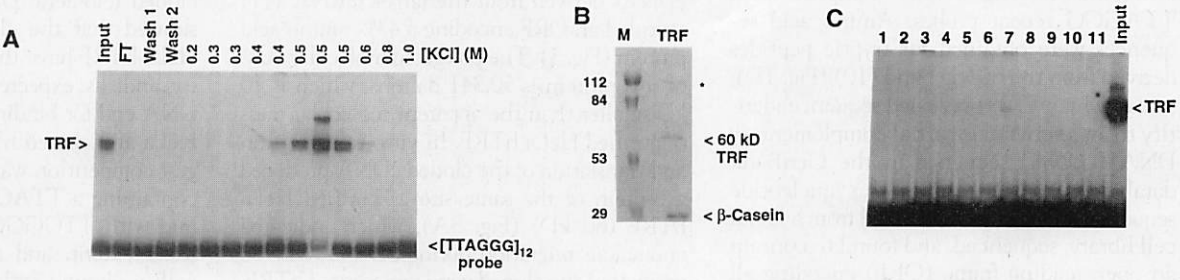

Fig. 1. Purification and identification of the 60-kD hTRF protein. (**A**) Specific DNA affinity chromatography of hTRF. Partially purified HeLa hTRF was applied to a column containing restriction fragments with the sequence $[TTAGGG]_{27}$ coupled to Streptavidin-agarose (*7*). Input, flow-through (FT), and the indicated KCl fractions were assayed for hTRF binding activity with the use of a $[TTAGGG]_{12}$ gel-shift probe. (**B**) Coomassie blue staining pattern of purified hTRF. The 60-kD TRF band is indicated. β-Casein was added to enhance hTRF activity in purified preparations (*7*). The asterisk at the right indicates a ~100-kD protein that is present in some of the hTRF preparations. Marker proteins (M) were prestained. (**C**) Recovery of hTRF activity by elution of the 60-kD protein from SDS-PAGE. Proteins from a gel similar to the one shown in (B) were eluted (*5*, *9*), and hTRF activity was assayed by gel shift with a $[TTAGGG]_{12}$ probe. Lanes 1 to 11 contain proteins isolated from successive gel slices covering the 120- to 20-kD range. Lane 7 contains proteins from the 55- to 65-kD range. (**D**) Analysis of hTRF tryptic peptides by chemical sequencing and laser-desorption mass spectrometry (*10*). Amino acids in lowercase were tentatively assigned; "x" indicates that no identification could be made (*27*). IY indicates calculated initial sequencing yields; *m/z* is the experimental mass of the peptide. [MH^+] denotes the theoretical average isotopic mass of the peptide (plus one proton), calculated from the cDNA-derived sequence (Fig. 3C). M_{ox} refers to methionine sulfoxide (singly oxidized methionine).

D

Peptide	Sequence	IY (pmol)	*m/z*	[MH^+] TRF
T7	EAEEVFEr	1.7	1009.2	1009.06
T8	TLDAqFENdEr	1.4	1337.9	1338.38
T10	TITsQDKPxxNxVxM	1.4	2672.0	2668.80 (M_{ox})
T11	ILLxYK	1.7	–	
T12	IqAIAVxm	0.65	1658.7	1659.80
T13	IFgDPNxxmpf	1.3	1408.5	1406.60 (M_{ox})
T20	xYVNYVLxEK	1.5	1201.4	1202.35
T26	QAxLxEEDK	1.1	–	
T29	TIYICQFTr	1.1	1363.0	1363.53

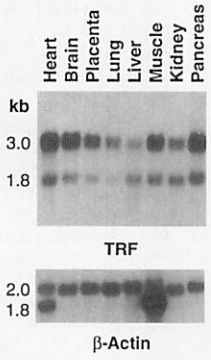

Fig. 2. Expression of hTRF mRNA in human tissues. The figure shows a Northern blot of polyadenylated RNAs (~2 μg per lane, Clontech) derived from the indicated tissues probed with a hTRF cDNA fragment encompassing most of the protein coding region. Human TRF is encoded by the 3-kb mRNA. The sequence of the 1.8-kb mRNA has not been determined. The bottom panel shows rehybridization of the same blot to a β-actin probe, which detects a 2-kb mRNA in all tissues and the muscle-specific 1.8-kb mRNA in heart and skeletal muscle.

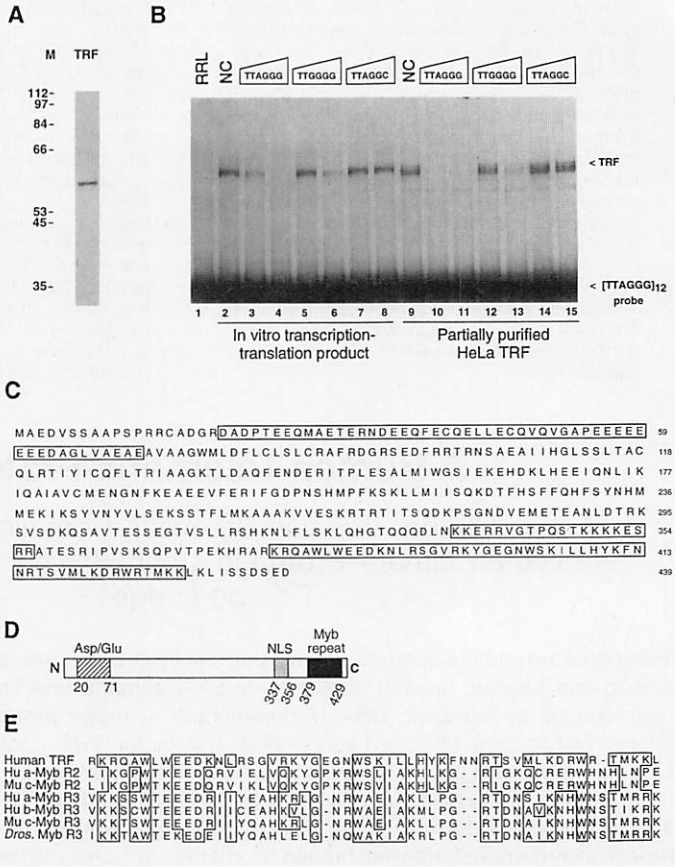

Fig. 3. Human TRF cDNA encodes a 60-kD telomeric DNA-binding protein with a Myb-type DNA-binding repeat and an acidic NH_2-terminal domain. (**A**) In vitro expression of the hTRF ORF yields a protein that migrates at ~60 kD. Shown is the [^{35}S]methionine-labeled in vitro transcription-translation product obtained with a reconstituted HeLa cDNA containing the complete TRF ORF. The position of molecular mass standards (M) is indicated. (**B**) In vitro–expressed hTRF binds telomeric DNA with the same sequence specificity as does HeLa hTRF. Unlabeled hTRF protein was produced in vitro [as in (A)] and assayed for binding to a $[TTAGGG]_{12}$ probe (lanes 2 to 8). Partially purified HeLa hTRF was assayed in parallel (lanes 9 to 15). Lane 1 contains a reaction with rabbit reticulocyte lysate (RRL) alone. Lanes 2 and 9 contain reactions without addition of competitor DNAs (NC). Lanes 3 to 8 and 10 to 15 contain binding reactions in the presence of 20 and 200 ng of circular plasmid DNAs containing stretches of 1 to 2 kb of tandemly repeated hexamers of the indicated sequences (*5*). (**C**) Conceptional translation of the ORF in hTRF cDNA (*11, 27*). The NH_2-terminal acidic region, two overlapping potential localization signals, and the region of homology to the Myb-type DNA-binding repeat are boxed. (**D**) Domain structure of hTRF (NLS, nuclear localization signal). (**E**) Alignment of hTRF to the Myb-type DNA-binding repeats of mammalian [Hu, human; Mu, mouse (murine)] and *Drosophila myb* proto-oncogenes (*11, 27*).

the basis of its ability to alter the mobility of a double-stranded DNA fragment containing the sequence $[TTAGGG]_{12}$ (*4*). Using this assay, we purified HeLa hTRF to near homogeneity by ion-exchange chromatography, by elution from columns containing nonspecific *Escherichia coli* DNA, and by fractionation over specific telomeric DNA resins (*7*) (Fig. 1A). Three independent preparations of purified hTRF contained a protein in the 60-kD apparent molecular mass range (Fig. 1B), which copurified with hTRF activity over a column containing double-stranded TTAGGG repeats (*8*). A ~100-kD protein was present in some but not all purified hTRF preparations (Fig. 1B) (*8*). Elution of the 60-kD protein from SDS–polyacrylamide gel electrophoresis (PAGE) (*9*) resulted in partial recovery of hTRF activity (Fig. 1C, lane 7), which indicates that a 60-kD polypeptide is sufficient for the formation of the hTRF complex with TTAGGG repeat probes. Amino acid sequences were obtained for tryptic peptides derived from the 60-kD band (*10*) (Fig. 1D), one of which (T29) revealed sequence identity to an anonymous partial complementary DNA (cDNA) sequence in the GenBank database. On the basis of this nucleotide sequence, cDNAs were isolated from a HeLa cell library, sequenced, and found to contain an open reading frame (ORF) encoding all sequenced peptides (*11*), as discussed below.

The hTRF cDNA hybridizes to two mRNAs of ~1.8 and ~3.0 kb that are expressed in a variety of human tissues (Fig. 2). Sequence analysis of three overlapping hTRF cDNAs derived from the larger mRNA (*11*) revealed an ORF encoding a 439–amino acid protein (Fig. 3). The predicted molecular mass of this protein is 50,341 daltons, which is 10 kD smaller than the apparent molecular mass of purified HeLa hTRF. In vitro transcription and translation of the cloned cDNA produced a protein of the same size as purified HeLa hTRF (60 kD) (Fig. 3A), which indicated anomalous migration during SDS-PAGE. To verify that the cloned gene represented hTRF, we used in vitro–expressed protein in mobility-shift assays with a $[TTAGGG]_{12}$ probe. The in vitro–expressed protein formed a complex with the telomeric DNA probe that comigrated with the largest of three closely migrating gel-shift complexes formed with HeLa hTRF (Fig. 3B). The two smaller hTRF complexes detected in HeLa extracts were never observed with in vitro–expressed hTRF. Whether the additional HeLa hTRF complexes resulted from modification in vivo or from alteration of the hTRF protein during isolation (for instance, by partial proteolytic degradation or dephosphorylation) is not known. Competition experiments with cloned telomeric DNAs in circular plasmids showed that the cloned hTRF protein and HeLa hTRF have the same sequence specificity and, as expected (*4*), do not require a DNA end for binding (Fig. 3B). For both the HeLa and cloned hTRF activities, the strongest competition was observed with a plasmid containing a TTAGGG repeat array; a plasmid with TTGGGG repeats competed to a lesser extent, and no competition occurred with a plasmid with TTAGGC repeats (Fig. 3B). These results demonstrated that the cloned cDNA encoded hTRF.

Comparison with the sequence information in the databases indicated that hTRF is a novel protein with three previously recognized sequence motifs (Fig. 3, C and D). hTRF contains two overlapping nucleoplasmin-type nuclear localization signals around position 350 and an NH_2-terminal region that is rich in aspartic and glutamic acid residues (*11*). The acidic domain of hTRF extends over a segment of 52 amino acids with 44% acidic residues and a calculated isoelectric point (pI) of 3.0. Although clus-

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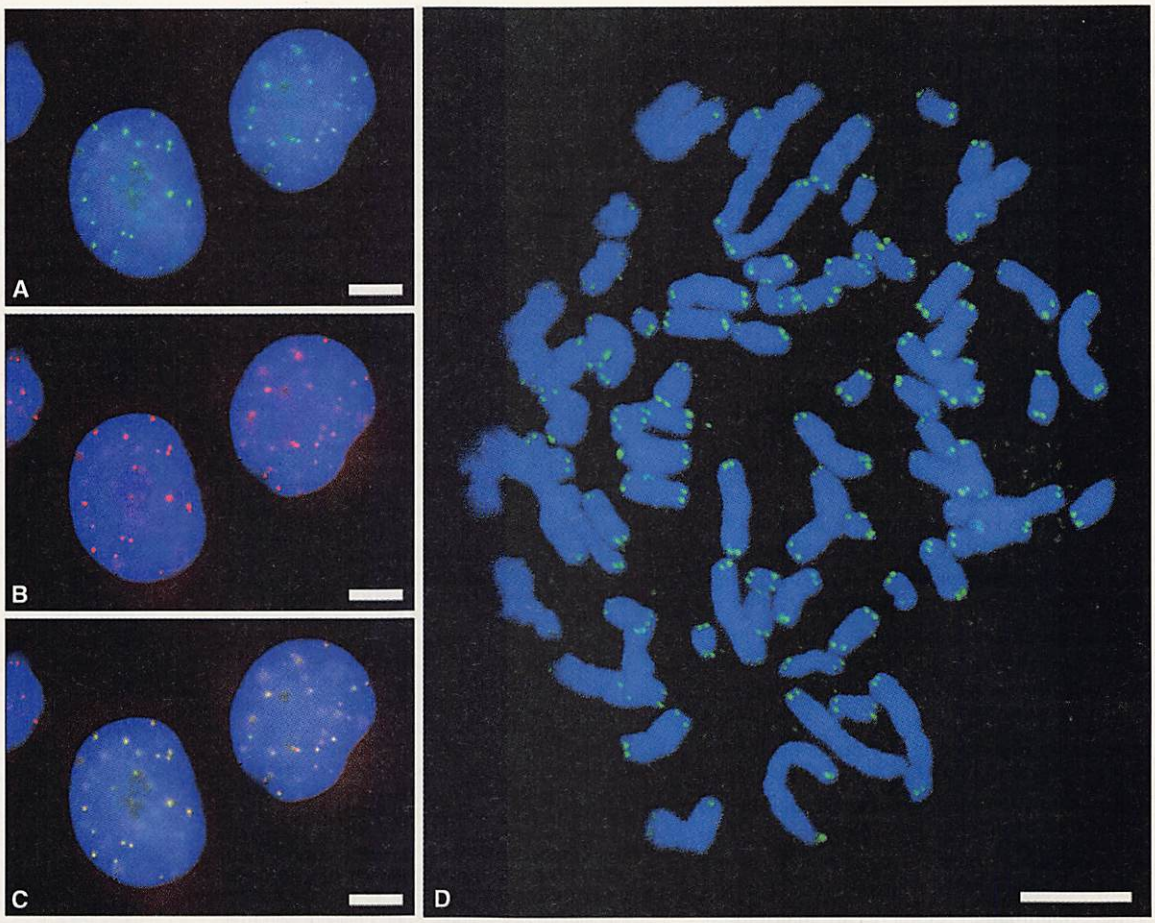

Fig. 4. Telomeric localization of TRF in human cells. (**A** through **C**) Colocalization of a FLAG epitope–tagged mTRF protein with telomeric DNA in interphase (*15, 16*). In (A), HeLa cells were transiently transfected with FLAG-mTRF expressed from the CMV promoter and labeled with an anti-FLAG mAb (M5) and FITC-conjugated donkey antibody to mouse IgG (green). In (B), telomeric DNA was visualized in the same nuclei by fluorescent in situ hybridization of a digoxigenin-labeled $[CCCUAA]_{27}$ RNA, followed by sheep anti-digoxigenin and TRITC-conjugated antibody to sheep IgG (red). In (C) is a superimposition of the images in (A) and (B). White and yellow indicate colocalization of the signals in (A) and (B). (**D**) Metaphase chromosomes of a HeLa cell expressing $[HA]_2$ epitope–tagged mTRF, labeled with an HA mAb (12CA5) and a FITC-labeled goat antibody to mouse IgG (green) (*15, 16*). In each panel, the DNA was stained with DAPI (blue). Scale bars, 5 μm.

ters of acidic residues have been primarily implicated in mediating protein-protein interactions during transcriptional regulation, they are also found in a number of proteins involved in chromosome function, including centromeric proteins, the p150 subunit of chromatin assembly factor I (CAF-I), and the largest subunit of the yeast origin recognition complex (ORC1) (*12*). The third recognizable motif in hTRF is a COOH-terminal region with strong homology to the DNA-binding repeats found in *myb* protooncogenes (Fig. 3D). These Myb-type DNA-binding repeats are ~50–amino acid stretches with three highly conserved tryptophan residues that form a helix-turn-helix (HTH) DNA-binding fold (*13*). Human TRF is most similar to the second and third Myb repeats in the mammalian and *Drosophila* Myb proteins. Unlike hTRF, these factors carry three tandem copies of this motif, two of which (R2 and R3) are sufficient for DNA binding (*13*). DNA-binding proteins carrying a single Myb repeat have been reported previously (*14*).

The nuclear and chromosomal location of TRF was determined by immunofluorescence microscopy of epitope-tagged mouse TRF (mTRF) protein expressed in transiently transfected HeLa cells (*15, 16*). Figure 4 shows that Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG)–tagged mTRF displayed a punctate pattern in interphase nuclei. A similar speckled distribution was found for hemagglutinin $[HA]_2$-tagged mTRF (*17*). A minority of the transfected cells showed homogeneous nuclear staining in addition to the speckled pattern, possibly because of greater amounts of mTRF expression (*17*). No specific patterns emerged in control experiments with untransfected cells or after omission of the primary antibody (*17*). Dual labeling experiments revealed that the speckled mTRF distribution coincided with telomeric DNA detected by TTAGGG repeat–specific fluorescent in situ hybridization (Fig. 4, B and C). All telomeric loci were found to contain mTRF, and, vice versa, all mTRF speckles were associated with telomeric DNA.

The chromosomal distribution of TRF was determined by expression of a fusion protein with two tandem HA epitopes at its NH_2-terminus. In metaphase chromosomes of stably transfected HeLa cells, $[HA]_2$-tagged mTRF was predominantly detected at chromosome ends (Fig. 4D). Mouse TRF appeared to be a common feature of all chromosome ends. Occasionally, telomeres without mTRF could be found, but the absence of mTRF did not appear to be specific for any one chromosome end and is most likely a result of difficulties in detection of the protein in metaphase spreads. Discrete localization of mTRF to internal loci was not observed. In some metaphase spreads, weak staining along the axis of all chromosomes accompanied the telomeric signals (*17*). However, the strongest signals were invariably observed at chromosome ends.

These results demonstrate that TRF is a telomeric protein in vivo. TRF occupies chromosome ends both in interphase and metaphase, consistent with its role in the telomeric complex. Indirect evidence had previously suggested that mammalian chromosome ends contain one or more telomere-specific proteins (*3–6, 18*). For instance, human telomeres display an altered chromatin structure and bind to the nuclear matrix (*18*). Moreover, formation of new human telomeres upon DNA transfection occurs only with telomere seeds that contain precise TTAGGG repeat arrays (*5*). The failure of other (closely related) repetitive sequences to form new telomeres suggested that telomere healing involved a specific telomeric DNA-binding protein (*5*). However, telomeric proteins had not been isolated from human cells or other vertebrates (*1, 6*). Our

results now demonstrate directly that human telomeres form a complex with a telomere-specific protein, hTRF. In agreement with a role for hTRF at telomeres, the sequence specificity of hTRF matches the sequence requirements for telomere formation in human cells (6). On the basis of the data presented here, we suggest that TRF binding is required for telomere function at the ends of chromosomes in human and other mammalian cells.

TRF is a double-stranded telomeric DNA-binding factor. Double-stranded telomeric DNA-binding proteins had previously been implicated in telomere function in budding yeasts. In *Saccharomyces cerevisiae*, Rap1p binds along the double-stranded telomeric TG_{1-3} tracts, where it regulates telomere length, chromosome stability, and telomeric position effects (*19*). Genetic alteration of the telomeric sequence of *Kluyveromyces lactis* has implicated a double-stranded telomeric DNA-binding protein in the regulation of telomerase (*20*). Although hTRF is not a Rap1p homolog, we note that the recently determined structure of a Rap1p-DNA complex reveals two Myb-type HTH motifs (*21*). Further structural and functional comparisons between these telomeric factors should be of interest. Several observations suggest that telomere maintenance in mammals, as in yeast, is subject to homeostasis (6). For instance, mammalian telomeres are maintained at constant length over the generations, and different mammals show species-specific telomere length (*22*). Moreover, telomerase expression in immortalized human cells does not lead to unlimited telomere elongation (*23*). A simple model to explain such phenomena invokes a factor (such as TRF) that binds along the length of the telomeric repeat array and modulates telomerase-mediated telomere elongation (6, *20*).

Human telomeres undergo programmed shortening in the soma (*16*, *24*). When grown in vitro, human cells enter a growth crisis at a stage when telomeres appear critically shortened and chromosome end fusions are frequent (*23*). Similarly, loss of telomeric DNA in human tumors has been suggested to contribute to genome instability in cancer (*2*, *25*). A mechanism to restore and maintain telomeres (such as activation of telomerase) may be required for tumor progression and cellular immortalization (*26*). Our findings raise the possibility that some of the deleterious consequences of telomere attrition may be caused by a failure of chromosome ends to bind protective telomeric proteins, including hTRF.

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7. Human TRF was isolated from nuclear extract derived from 9.2×10^{11} HeLa cells containing 4 g of protein and ~650 pmol of hTRF (1 pmol of TRF is defined as the amount of hTRF required to bind 1 pmol of probe) over a series of ion exchange and affinity columns. Each column was loaded in dialysis buffer (buffer D; 100 mM KCl, 20 mM Hepes, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.1% NP-40) and eluted with increasing KCl concentration in the same buffer without glycerol. Activity was determined by gel-shift assay with the use of a 142–base pair (bp) restriction fragment containing the hTRF binding site $[TTAGGG]_{12}$ (*4*). In the course of this work, it was noted that fractionation of partially purified hTRF over a column containing double-stranded $[TTAGGG]_{27}$ DNA resulted in dramatic loss of gel-shift activity of the factor. No such loss of activity was observed during fractionation of crude nuclear extract over the same resin. The lost activity of highly purified hTRF could be recovered in the 0.5 M KCl fractions by the addition of β-casein, *E. coli* lysate, guanosine 3′,5′-monophosphate phosphodiesterase, or calf thymus high mobility group–1, but not by the addition of bovine serum albumin, collagen, γ-globulin, α-transducin, or glutathione-S-transferase. By contrast, hTRF activity in crude HeLa nuclear extract was not enhanced by casein or *E. coli* lysate. Restoration of DNA-binding activity by casein and *E. coli* lysate has also been observed with highly purified fractions of the yeast centromeric binding factor CBF3 [J. Lechner and J. Carbon, *Cell* **64**, 717 (1991)]. For the final purification of hTRF, the following columns were used: DEAE-Fractogel 650 (EM Separations, Gibbstown, NJ) with hTRF eluting at 0.2 to 0.3 M KCl, P11 (Whatman, Marchstone, Kent, England) with hTRF eluting at 0.6 M KCl, and CM-Sepharose (Pharmacia) with hTRF eluting at 0.4 to 0.5 M KCl. Subsequently, hTRF was fractionated on Streptavidin-agarose beads carrying biotinylated, HinfI–digested *E. coli* chromosomal DNA from which hTRF eluted at 0.2 to 0.3 M KCl, followed by an affinity column composed of Streptavidin beads with biotinylated Asp718 restriction fragments containing $[TTAGGG]_{27}$. Human TRF eluted from this column at 0.5 M KCl (Fig. 1). The active fraction was supplied with β-casein and fractionated again by batch elution on nonspecific and specific (TTAGGG) DNA-containing resins. The final sample was precipitated with 20% trichloroacetic acid and 0.015% deoxycholate, fractionated on SDS-PAGE, and transferred to nitrocellulose. The sequenced hTRF preparation was composed of two polypeptides of ~60 kD; the larger protein was present at subpicomolar amounts and did not yield sequence information. Its relation to hTRF remains to be determined.
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10. Peptides were generated from the nitrocellulose-bound hTRF protein by in situ tryptic digestion [H. Erdjument-Bromage *et al.*, *Protein Sci.* **3**, 2435 (1994)] and fractionated by reversed-phase high-performance liquid chromatography [C. Elicone *et al.*, *J. Chromatogr.* **676**, 121 (1994)], and selected peak fractions were analyzed by a combination of automated Edman degradation and laser-desorption mass spectrometry [P. Tempst, S. Geromanos, C. Elicone, H. Erdjument-Bromage, *Methods* **6**, 248 (1994)]. Peptide sequences were compared to entries in various sequence databases with the use of the National Center for Biotechnology Information (NCBI) BLAST program (S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990)]. Average isotopic masses of predicted peptides were summed from the cDNA-derived sequence with the use of ProComp software (version 1.2; P. C. Andrews, University of Michigan, Ann Arbor, MI).
11. TRF cDNAs were isolated from a HeLa cDNA library (Stratagene) by screening with a 33-nucleotide DNA probe that represents the region of homology of T29 peptide sequence to an anonymous cDNA in GenBank (accession number Z19923). Three overlapping cDNAs, together spanning 2684 bp, were sequenced (GenBank accession number U40705). The sequence contains a single long ORF of 439 amino acids starting with an AACATGG initiating codon, which is favorable for translation initiation [M. Kozak, *Nucleic Acids Res.* **15**, 8125 (1987)]. The 3′ end of the TRF cDNA contains an AATAAA polyadenylate [poly(A)] addition signal 18 bp upstream of a poly(A) stretch. The TRF ORF amino acid sequence was compared to proteins in the nonredundant database with the use of the program BLASTP (*10*). The nuclear targeting signals are of the nucleoplasmin type [J. Robbins, S. M. Dilworth, R. A. Laskey, C. Dingwall, *Cell* **64**, 615 (1991)]. Alignment to the Myb proteins was achieved with the program Clustalw 1.5; a SeqVu 1.0.1 display and Rao rules for conservative amino acid changes were used.
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15. A cDNA for the mouse homolog of hTRF was isolated by cross-hybridization to the human cDNA (D. Broccoli, L. Chong, T. de Lange, unpublished data). The ORF in this cDNA was fused to a FLAG epitope. HeLa cells with long telomeres in the 15- to 30-kb size range [T. de Lange, *EMBO J.* **11**, 717 (1992)] were electroporated with a construct containing the FLAG-mTRF gene expressed from the cytomegalovirus (CMV) promoter and grown for 24 hours on Alcian Blue–coated cover slips. Cells were washed in phosphate-buffered saline, fixed in 2% formaldehyde in phosphate-buffered saline for 10 min, and permeabilized with 0.5% Nonidet P-40 in phosphate-buffered saline for 20 min. In situ hybridization was carried out essentially as described [O. C. M. Sibon *et al.*, *Histochemistry* **101**, 223 (1994)] with the use of a digoxigenin-labeled $[CCCUAA]_{27}$-repeat RNA probe made by in vitro transcription of plasmid pTH5 (*16*) using 11-digoxigenin–uridine 5′-triphosphate (Boehringer). Cells were incubated with sheep antibody to digoxigenin (anti-digoxigenin) (Boehringer) and mouse monoclonal antibody (mAb) M5 (Eastman Kodak) against the FLAG epitope, followed by tetramethyl rhodamine isothiocyanate (TRITC)–conjugated donkey antibody to sheep immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)–conjugated donkey antibody to mouse IgG (both from Jackson ImmunoResearch Labs). DNA was stained with 4,6-diamino-2-phenylindole (DAPI, 0.2 μg/ml). Cells were embedded in *p*-phenylene diamine (1 mg/ml) (Sigma) and 90% glycerol in phosphate-buffered saline (pH 7.8). After omission of the $[CCCUAA]_{27}$ probe or anti-digoxigenin, or when nuclear DNA was not denatured, only the FITC signal was found. In untransfected cells or after omission of mAb M5, only the TRITC signal was observed. Formaldehyde-fixed chromosome spreads were prepared essentially as described [D. A. Compton, T. J. Yen, D. W. Cleveland, *J. Cell Biol.* **112**,1083 (1991)]. HeLa cells were stably transformed by calcium phosphate coprecipitation of a construct containing a $[HA]_2$-tagged mTRF gene expressed from the CMV promoter and a neomycin-selectable marker (pRC/CMV, Invitrogen). A clonal G418-resistant cell line that expressed $[HA]_2$-tagged mTRF was isolated, treated with colcemid (0.1 μg/ml, 90 min), harvested by trypsinization, hypotonically swollen in 10 mM tris (pH 7.4), 10 mM NaCl, and 5 mM $MgCl_2$, and sedimented onto Alcian Blue–treated cover slips for 15 s at 3000 rpm in a Sorvall RT6000B tabletop centrifuge. Chromosome spreads were immediately fixed in 2% formaldehyde in phosphate-buffered saline,

permeabilized in 0.5% Nonidet P-40 in phosphate-buffered saline, and labeled with mouse mAb 12CA5 followed by FITC-labeled goat antibody to mouse IgG (Jackson ImmunoResearch Labs). DNA was stained with DAPI (0.2 μg/ml). Images of interphase nuclei were obtained with a Zeiss Axioplan microscope equipped with a Kodak DCS-200 digital camera. Images were noise-filtered with a 3 × 3 median filter, corrected for background with nontransfected cells as a reference, and merged to obtain triple labeling images using Adobe Photoshop. Chromosome spreads were photographed on Kodak Gold II 400 ISO film and converted to digital images with a Nikon slide scanner, after which FITC and DAPI images were corrected for background and superimposed.

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Conserved Initiator Proteins in Eukaryotes

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The origin recognition complex (ORC), a multisubunit protein identified in *Saccharomyces cerevisiae*, binds to chromosomal replicators and is required for the initiation of cellular DNA replication. Complementary DNAs (cDNAs) encoding proteins related to the two largest subunits of ORC were cloned from various eukaryotes. The cDNAs encoding proteins related to *S. cerevisiae* Orc1p were cloned from the budding yeast *Kluyveromyces lactis*, the fission yeast *Schizosaccharomyces pombe*, and human cells. These proteins show similarity to regulators of the S and M phases of the cell cycle. Genetic analysis of *orc1*$^+$ from *S. pombe* reveals that it is essential for cell viability. The cDNAs encoding proteins related to *S. cerevisiae* Orc2p were cloned from *Arabidopsis thaliana*, *Caenorhabditis elegans*, and human cells. The human ORC-related proteins interact in vivo to form a complex. These studies suggest that ORC subunits are conserved and that the role of ORC is a general feature of eukaryotic DNA replication.

The replicon model for the initiation of DNA replication postulates that for DNA synthesis to occur, an initiator protein is required for recognition of a specific replicator sequence in the chromosome (*1*). In this model, recognition of the replicator by the initiator protein determines the location of an origin of DNA replication. Data from studies of prokaryotes and eukaryotic viruses support this hypothesis and suggest that the mechanism of initiation of DNA replication is conserved in eukaryotes. The initiation of DNA replication in eukaryotic cells is tightly controlled during the cell cycle and throughout development to ensure that duplication of the genome occurs only once per cell cycle. Thus, initiation is a key regulatory step in DNA replication.

In eukaryotes, the nature of initiator proteins and replicator elements remains unclear (*2*). Attempts to define precisely the origins of DNA replication have been largely unsuccessful. A notable exception is the yeast *Saccharomyces cerevisiae*, in which the origins of DNA replication have been physically mapped (*3*). Autonomously replicating sequences (ARS) have been characterized at the molecular level, revealing a modular structure (*4*, *5*). All ARS elements contain an essential ARS consensus sequence (ACS) and other elements that together are required for ARS function. In addition to characterized replication origins, an initiator protein complex has been isolated (*6*). ORC was identified by its ability to recognize and bind to the ACS in an adenosine triphosphate (ATP)–dependent manner. ORC recognizes a bipartite sequence within the replicator (*6*, *7*) and is bound to the DNA throughout the cell cycle (*8*). In cooperation with other cell cycle proteins, ORC determines the frequency of initiation in the *S. cerevisiae* genome (*9*–*12*).

ORC is a multisubunit protein complex consisting of six polypeptides with apparent molecular mass ranging from 50 to 120 kD. The genes encoding all the subunits have been cloned (*11*–*14*) and all are essential for cell viability. All six genes encode novel proteins, although *ORC1* and *ORC5* encode proteins that have regions of sequence similarity that are present in previously identified proteins. *ORC1* encodes the largest subunit of ORC, and its protein product is related to two other known yeast proteins (*13*). The protein most related to Orc1p is Sir3p, a protein involved in transcriptional silencing (*15*). Although these proteins are related over their entire length, the most striking similarity is found in the first 220 amino acids. A pairwise comparison shows that the proteins are 50% identical and 63% similar over this region. Orc1p is also related to two cell division cycle (CDC) regulators of initiation of DNA replication and control of the G_2- to M-phase transition, *S. cerevisiae* Cdc6p and *Schizosaccharomyces pombe* Cdc18p (*16*). A 270–amino acid region in Orc1p (residues 449 to 717) contains a purine nucleotide-binding motif (*17*) that is present in both Cdc6p and Cdc18p. In addition to the canonical P-loop and A-loop necessary for nucleotide metabolism (*17*), there are sequences flanking these nucleotide-binding motifs that are present only in Orc1p, Cdc6p, and Cdc18p (*13*). Orc1p is 50% identical to Cdc6p and Cdc18p across this region. We refer to this domain as the CDC–nucleoside triphosphate–binding (CDC-NTP) domain. Although Sir3p shares sequence homology with Orc1p across this domain, key residues essential for nucleotide binding are absent from Sir3p (*13*).

The nature of replicators in metazoan species is not clear, and in *Xenopus* early embryos their very existence has been questioned (*18*). To begin to address whether the replicon model applies to cell chromosome replication in higher eukaryotes, we identified genes related to *ORC1* in humans and other organisms (Fig. 1). We used a polymerase chain reaction (PCR) strategy, with primers based

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on regions conserved between Orc1p and Sir3p, and identified a gene in the related budding yeast *Kluyveromyces lactis* whose product KlOrc1p is related to Orc1p (*19*). KlOrc1p is 50% identical and 68% similar to *S. cerevisiae* Orc1p (ScOrc1p) overall (Fig. 1B). Within KlOrc1p are two regions that are highly related to ScOrc1p. The NH_2-terminal domain (NTD), with high identity between Sir3p and Orc1p, is conserved in KlOrc1p; the proteins are 32% identical and 43% similar over residues 1 to 220. However, the conservation between KlOrc1p and ScOrc1p is most striking in the CDC-NTP domain (69% identical and 81% similar; Fig. 1A). To identify other *ORC1*-related genes, we used a DNA probe from *S. cerevisiae ORC1* containing the CDC-NTP domain and isolated a related gene from *S. pombe* by low-stringency hybridization (*20*). Sequence analysis revealed that *S. pombe* Orc1p (SpOrc1p) and ScOrc1p are 30% identical overall (Fig. 1B). Like KlOrc1p, SpOrc1p is most related to ScOrc1p across the CDC-NTP domain; this region is 46% identical and 61% similar to ScOrc1p (Fig. 1A). In contrast with KlOrc1p, the NTD of Orc1p and of Sir3p is absent in SpOrc1p. Indeed, the first 200 amino acids of *S. cerevisiae* Orc1p are not essential for viability but are required for mating type gene repression (*13*). The absence of this domain in a species that has evolutionarily diverged from the budding yeasts suggests that specialized mating type control and silencing are not conserved.

The identification of *ORC1*-related genes in both budding and fission yeasts suggests that Orc1p is conserved in higher eukaryotes. To investigate this, we again used PCR to identify a human *ORC1*-related gene. The PCR primers were designed based on conserved residues in the CDC-NTP domain that is present in ScOrc1p, KlOrc1p, and SpOrc1p; and reverse transcription PCR (RT-PCR) reactions were then carried out on human RNA. An amplified DNA fragment similar to *ORC1* was generated and used as a probe to isolate full-length cDNAs (*21*). Sequence analysis revealed that this gene product was related to Orc1p, Cdc6p, and Cdc18p (Fig. 1A). A BLAST database search comparing the human gene product with both Orc1p and Cdc6p or Cdc18p showed the protein to be more closely related to Orc1p ($P = 7.4 \times 10^{-52}$ for Orc1p, compared with $P = 3.6 \times 10^{-10}$ for Cdc6p) (*21*). *Homo sapiens* Orc1p (HsOrc1p) is 27% identical to ScOrc1p overall, but is 44% identical and 60% similar to ScOrc1p across the CDC-NTP domain (Fig. 1A). The Orc1 proteins can be distinguished from Cdc6p and Cdc18p by sequences in the CDC-NTP domain that are conserved among Orc1 proteins but not among Cdc18p or Cdc6p (for example, residues upstream and downstream of CDC-NTP box 4). In addition, there are shorter stretches of conserved residues both upstream of the CDC-NTP domain and downstream, extending to the end of the proteins (Fig. 1B).

Genetic analysis of *ScORC1* shows that the gene is essential for cell viability (*13*). To test whether *S. pombe orc1*$^+$ is essential, we created a null allele of *orc1*$^+$ by replacing a 1.4-kb fragment of the coding sequence with the *ura4*$^+$ gene (*22*). Southern (DNA) blot analysis confirmed that stable Ura$^+$ transformants carried one wild-type allele and one disrupted allele. Sporulation of the heterozygous diploid produced two viable *ura*$^-$ spores and two nonviable spores, demonstrating that *orc1*$^+$ is essential (*23*). The lethal phenotype of the disruption could be rescued by a plasmid containing a genomic fragment of *orc1*$^+$ or an influenza

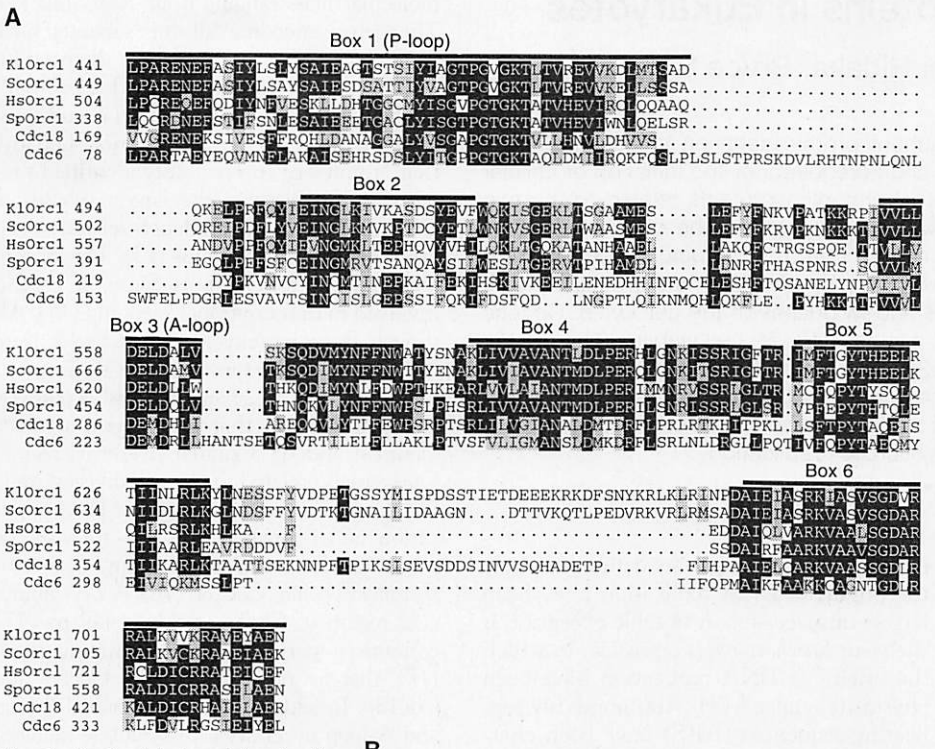

Fig. 1. Orc1-related proteins in diverse organisms (*33*). (**A**) Multiple alignment of Orc1 proteins with Cdc6p and Cdc18p across the CDC-NTP region. KlOrc1, ScOrc1, HsOrc1, and SpOrc1 indicate Orc1 proteins from *K. lactis, S. cerevisiae*, human cells, and *S. pombe*, respectively, and Cdc18 and Cdc6 are Cdc18p from *S. pombe*, and Cdc6p from *S. cerevisiae*, respectively. Identical amino acids conserved among three or more sequences are in black; identical amino acids conserved among two sequences, or similar amino acids conserved among three or more sequences, are shaded gray. Amino acid numbers are shown on the left. Boxes 1 through 6 show regions of high similarity in Orc1p, Cdc18, and Cdc6. (**B**) Comparison of Sir3p; Orc1 proteins from *S. cerevisiae, K. lactis, S. pombe*, and human cells; Cdc6p; and Cdc18p. The NTD conserved among Sir3p, ScOrc1p, and KlOrc1p is indicated by a hatched box. The CDC-NTP motif is indicated by a shaded box, with the integral nucleotide binding site represented by a dark, shaded box. The percent identity of Orc1-related proteins with respect to ScOrc1p is shown on the right. Regions of conservation among Orc1 proteins and Sir3p or Cdc6 and Cdc18p at the COOH-terminus are indicated by black lines; conservation among Orc1 proteins is indicated by gray lines. aa, amino acid.

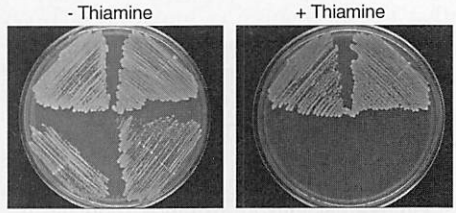

Fig. 2. *Schizosaccharomyces pombe orc1*$^+$ is essential for viability. Wild-type cells (top right quadrant), *orc1::ura4*$^+$ cells containing either a genomic *orc1*$^+$ under its native promoter (top left quadrant), or a plasmid containing the *orc1*$^+$ cDNA under the control of the *nmt1* promoter (bottom) were grown in the absence (−) or presence (+) of thiamine at 30°C. Two independent colonies containing *nmt1-orc1*$^+$ were tested.

virus hemagglutinin epitope–polyhistidine–tagged *orc1*$^+$ cDNA under the control of the inducible *nmt1* promoter (*24*). The *orc1* gene disrupted mutant cells carrying *orc1*$^+$ on a plasmid were grown in the absence or presence of thiamine (*25*). Under nonrepressing conditions (in the absence of thiamine), *nmt1-orc1* cells were viable, but in the presence of thiamine, no viable cells were recovered (Fig. 2). The presence or absence of thiamine had no effect on cells that contained a genomic *orc1*$^+$ under the control of its own promoter. Although *KlORC1* and *SpORC1* are related to *ScORC1*, neither can functionally substitute for *ScORC1* (*23*). Analysis of *S. pombe orc1*$^+$ is in preparation (*26*).

As Orc1p in *S. cerevisiae* is part of a multiprotein complex, the identification of related proteins in diverse eukaryotes suggests that additional ORC proteins are conserved. Database searches revealed expressed sequence tags of partial cDNAs from *A. thaliana* and *C. elegans* that encoded amino acids related to *S. cerevisiae* Orc2p. The partial gene fragments were used as probes to isolate full-length cDNAs from both *A. thaliana* (*27*) and *C. elegans*. (*28*). Comparison of the full-length proteins with *S. cerevisiae* Orc2p revealed short stretches of conservation (Fig. 3). To identify an *ORC2*-related gene in human cells, primers based on these conserved amino acids were used in RT-PCR reactions containing human RNA. DNA fragments that were related to *ORC2* were obtained and used as probes to isolate a full-length cDNA (*29*). Alignment of AtOrc2p, HsOrc2p, CeOrc2p, and ScOrc2p shows that the proteins are 23 to 31% identical overall (Fig. 3). We and others have also isolated a full-length cDNA encoding *Drosophila* Orc2p and shown it to be similar to those we describe here (*23*, *30*). The sizes of the Orc2 proteins are highly variable, and this variability is seen predominantly in the NH_2-termini. The region conserved among the four species is localized to the COOH-terminus. The Orc2 proteins lack any sequence characteristics that are shared with other previously identified proteins. Unlike Orc1 proteins, in which the similarity across the CDC-NTP motif spans 270 amino acids, the regions conserved among the Orc2-related proteins are short and are separated by less well-conserved sequences.

The *S. cerevisiae* Orc1p and Orc2p interact with four additional subunits in vivo to form a functional initiator complex, suggesting that HsOrc1p and HsOrc2p would similarly associate with each other as part of a multiprotein complex. To test this, a T7 epitope–tagged HsOrc1p was transiently overexpressed in human 293 cells (*31*). Rabbit polyclonal antisera raised to glutathione-S-transferase (GST)–fused HsOrc2p were used to immunoprecipitate endogenous HsOrc2p from extracts prepared from transfected cells (*31*). Immunoblot analysis with a monoclonal antibody specific for the epitope detected HsOrc1p in samples containing HsOrc2p antisera (Fig. 4, lanes 5 and 6) but not from immunoprecipitations using preimmune sera (Fig. 4, lane 4) or from extracts of mock-transfected cells (Fig. 4, lane 3). The stronger signals between 30 and 50 kD represent cross-reactivity to immunoglobulins. These data demonstrate that HsOrc1p and HsOrc2p are part of a complex in vivo. Moreover, the endogenous HsOrc1p and HsOrc2p cofractionate during column chromatography (*23*).

Our data demonstrate that two subunits of the yeast initiator complex are conserved in different eukaryotes, including complex vertebrates. Thus, it is likely that other subunits of the protein complex are conserved. Recent identification of a multiprotein complex from *Drosophila* that contains two ORC-related proteins (*30*) further supports this hypothesis. The high degree of conservation of the CDC-NTP domain among the Orc1-related proteins also suggests that the nucleotide requirement for DNA binding is also conserved. Data from *S. cerevisiae ORC1* show that an intact nucleotide-binding mo-

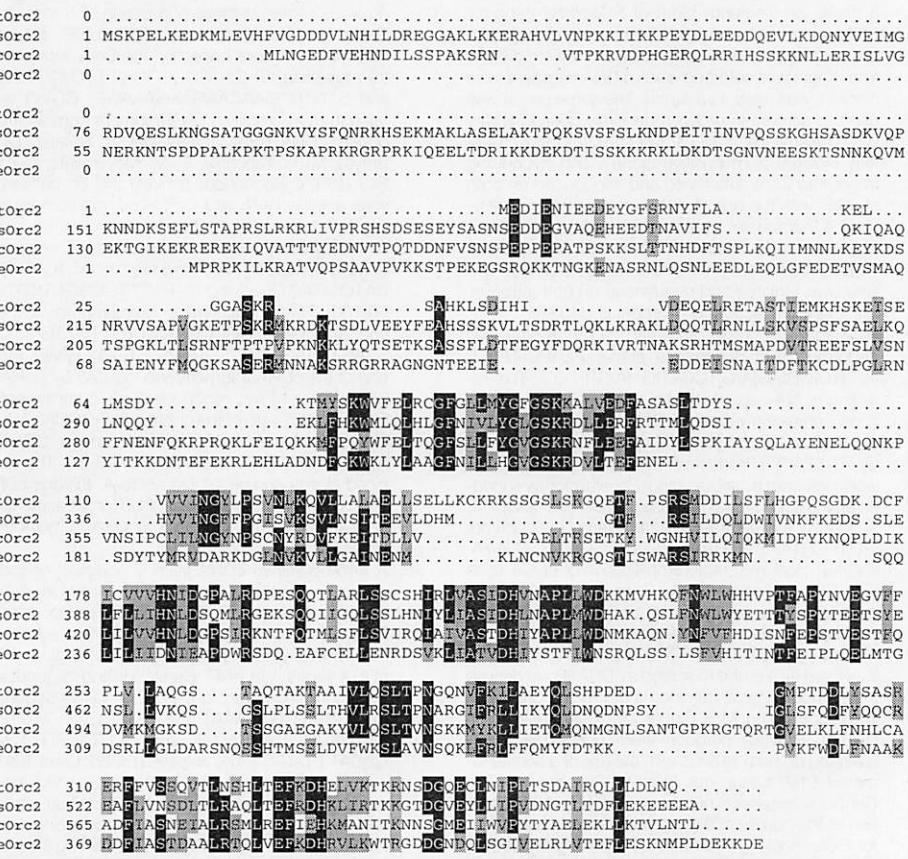

Fig. 3. Multiple alignment of Orc2 proteins in various organisms (*33*). AtOrc2, HsOrc2, ScOrc2, and CeOrc2 represent Orc2 polypeptides of *A. thaliana*, humans, *S. cerevisiae*, and *C. elegans*, respectively. Identical amino acids conserved among three or more species are in black; gray shading indicates similar amino acids conserved among three or more sequences. Amino acid numbers are shown on the left.

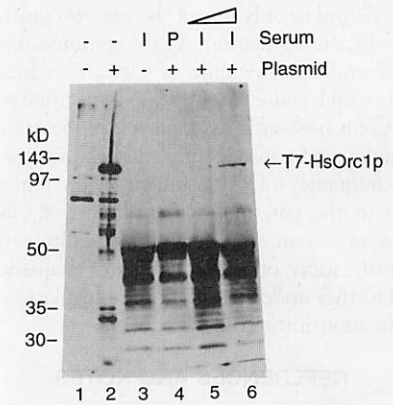

Fig. 4. Association of HsOrc1p and HsOrc2p in vivo. Human 293 cells were transiently transfected with a plasmid expressing T7-HsORC1 under the cytomegalovirus promoter or were mock-transfected. Whole-cell extracts of mock-transfected (lane 1) or transfected (lane 2) cells were prepared and used for immunoprecipitations. Immune complexes were collected, separated by SDS-PAGE, and transferred to nitrocellulose membrane. Immunoblot analysis was done with a monoclonal antibody to the T7 epitope. Lane 3, mock-transfected extract incubated with 4 μl of immune (I) sera. T7-HsORC1 extracts were incubated with 4 μl of preimmune (P) sera (lane 4) or with increasing amounts of antibodies to HsOrc2p (1 μl, lane 5; 4 μl, lane 6), as indicated by the triangle. T7-HsOrc1p is indicated by an arrow.

tif is essential for cell viability (*13*). Moreover, initiator proteins in bacteria, bacteriophages, and viruses commonly show a dependence on nucleo- tide binding for function (*32*). Although the exact role of ATP or GTP in Orc1p function has not yet been determined, the finding that the nucleotide-binding motif is conserved through evolution suggests that its role is important.

Genetic data support the idea that ORC in association with other replication proteins determines the frequency of origin firing in the genome (*10*). These data suggest that the ORC complex is conserved [our data and (*30*)] and that initiation of replication in metazoan species is likely to be DNA sequence–specific. This sequence specificity for establishment of replication origins may be subject to developmental regulation. For instance, the replication of foreign DNAs in *Xenopus* early embryos or extracts is subject to cell cycle regulation; however, initiation occurs in an apparently sequence-independent manner (*18*). If ORC is required for replication in the early embryo, how might this be reconciled with an apparent lack of DNA sequence specificity? Under stringent binding conditions, ORC binds to and requires a bipartite sequence element in yeast replicators (*7*). In contrast, the stringency of ORC binding to the origin DNA is relaxed as the competitor DNA concentration is decreased. By analogy, a high concentration of ORC relative to origin DNA present in an early embryonic cell (*30*) could cause sequence-specific initiation to be relaxed, because ORC would bind to only one of the two recognition sites in the replicator. As the genome replicates and the stockpile of ORC is reduced, this would cause an increased specificity of ORC for high-affinity, bipartite replicator sequences, and consequently cause a decrease in the frequency of initiation of DNA replication in the genome. The isolation of ORC from metazoan species should facilitate the identification of these replicator sequences and further understanding of the regulation of replication initiation.

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19. PCR reactions were carried out with *K. lactis* genomic DNA and degenerate oligonucleotides ORC1–3N (5′-AC[A/C/T]TATTC[A/C/T]GC[A/C/T]TATATGAT[A/C/T] CA-3′) and ORC1–2C(5′-AAGTTC[T/G/A]GC[T/G/A] GT[T/C]AA[G/A]TA[T/C]AA[T/G]TC-3′) based on regions of conservation between Orc1p and Sir3p. A 230–base pair (bp) amplified product related to *S. cerevisiae* Orc1p was used as a probe to screen a *K. lactis* genomic plasmid library [M. J. R. Stark and J. S. Milner, *Yeast* **5**, 35 (1989)]. Two positive clones were isolated and were sequenced on both strands by standard dideoxy sequencing. The GenBank accession number is U40151.
20. An 800-bp Eco RI–Pvu II *S. cerevisiae ORC1* fragment containing the CDC-NTP domain was used as a probe on Southern blots of *S. pombe* genomic DNA under reduced hybridization stringency. Hybridizing fragments were detected that were distinct from fragments detected when a DNA fragment from *cdc18*$^+$ was used as a probe. The same probe was used to screen an ordered genomic cosmid library [T. Mizukami *et al.*, *Cell* **73**, 121 (1993)]. Cosmid DNA was isolated from positive clones, and hybridizing fragments were subcloned and sequenced on both strands with the use of dideoxy sequencing. A 2.0-kb Eco RI fragment was used as a probe to screen a cDNA library [J. Field *et al.*, *Mol. Cell Biol.* **8**, 2159 (1988)]. Positive clones were plaque-purified, and DNA was isolated and sequenced on both strands. The GenBank accession number is U40378.
21. Reverse transcription of human 293 total RNA was performed with degenerate primer PO1PCR7 (5′-[C/T]TCIGGIAG[A/G]TCCATIGT[A/G]TT-3′). The resulting cDNA was used as a template in PCR reactions containing primers PO1PCR7 and PO1PCR3 (5′-GT[C/G/T]CG[C/G/T][C/A]TIGA[C/T]GA[A/G]CTI- G A-3′). Products of the correct predicted size were analyzed by sequencing. Several clones encoded amino acids similar to those of *S. cerevisiae* ORC1. To identify a larger fragment, an internal exact primer (PO1PCR14, 5′- GGACCTTCTGTGGACTCACAAA-3′) was used in 3′ rapid amplification of cDNA ends (RACE) reactions containing 3′ anchor primer and 3′ adaptor primer (Gibco-BRL). Sequence analysis of amplified products revealed fragments with high homology to *S. cerevisiae ORC1*. One RACE product was used as a probe to screen a cDNA library derived from a human teratocarcinoma cell line [J. Skowronski, T. G. Fanning, M. F. Singer, *Mol. Cell. Biol.* **8**, 1385 (1988)]. Positive plaques were purified and sequenced on both strands with the use of a semiautomated DNA sequencer (Applied Biosystems). The GenBank accession number is U40152. A comparison of the human cDNA against the National Center for Biotechnology Information (NCBI) databases was done with the BLAST algorithm [S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990)].
22. An *S. pombe orc1* knockout construct was made by digestion of pKG9, which contains a 6.0-kb Pst I genomic fragment isolated from the *S. pombe* cosmid library screen, with Not I and Sma I and filling in with Klenow to destroy the polylinker Spe I site. The plasmid was then digested with Spe I, and a 1.4-kb Spe I fragment (encoding two-thirds of the open reading frame) was replaced with the 1.8-kb *ura4*$^+$ gene. This construct was digested with Pst I and used to transform diploid strain SP826 (*h*$^{+N}$/*h*$^{+N}$ *ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18*) by standard methods [S. Moreno, A. Klar, P. Nurse, *Methods Enzymol.* **194**, 796 (1991)]. Stable Ura$^+$ transformants analyzed by Southern blot carried one copy of the *orc1* disruption and one copy of the wild-type gene. After conversion of the *h*$^{+N}$ allele to *h*^{90}, tetrads from these diploids were dissected and found to segregate into two viable and two nonviable spores.
23. K. Gavin, M. Hidaka, B. Stillman, unpublished data.
24. For insertion of a HA-HIS6 epitope cassette immediately preceding the initiator methionine, a Bam HI site was introduced by PCR into pKG04, which contains a full-length *S. pombe orc1*$^+$ cDNA cloned into the Not I site of pSK- (Stratagene). In addition, an Nde I site was introduced at the initiator methionine to facilitate cloning into pREP1 [K. Maundrell, *Gene* **123**, 127 (1993)]. The resulting amino acid sequence at the NH_2-terminus is MGSYPYDYAHHHHHHG-SPRRKSLR . . . (*33*). The resulting plasmid (pKG14) was digested with Not I, blunt-ended with Klenow polymerase, then digested with Nde I. The vector pREP1 was digested with Sma I and Nde I and the tagged *orc1* fragment was cloned into the Nde I–Sma I sites to make pKG15.
25. Strain YB0243 (*h*^{90}/*h*$^{+N}$ *orc1::ura4/orc1*$^+$ *ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18*) was transformed with pKG15 or pKG26 [a 6-kb Hind III genomic fragment in pWH5 isolated from an *S. pombe* genomic library [M. Caligiuri and D. Beach, *Cell* **72**, 607 (1993)]] and sporulated on selective media. Colonies were streaked onto pombe minimal media containing adenine with or without thiamine.
26. K.A. Gavin and B. Stillman, unpublished data.
27. A 344-bp partial cDNA from *A. thaliana* encoding amino acids similar to those of a region of *S. cerevisiae* Orc2p was identified in the NCBI dbEST database (no. 1443). Primers based on the DNA sequence in the database (5′-CTTGCCCGGCTTTCTTCTTG-3′ and 5′-GTGTGAACAAGAAGAAAGC- CG-3′) were used to PCR-amplify a DNA fragment from an *Arabidopsis* cDNA library [D. Weigel, J. Alvarez, D. R. Smyth, M. F. Yanofsky, E. M. Meyerowitz, *Cell* **69**, 843 (1992)]. Sequences flanking this on both sides were amplified with nested PCR primers complementary to each end of the fragment, and with a primer complementary to vector sequences. The primers complementary to the 5′ end are 5′-CACTCTTGAC-CATCGGAGTT-3′ and 5′-TGCTTCCGGCTCGTAT-GTTGTGTG-3′. The primers complementary to the 3′ end are 5′-CTTGCCCGGCTTTCTTCTTG-3′ and 5′-AGTCAAGTGACTTTAAA- CTC-3′. Finally, the 5′ end of the cDNA fragment was isolated by 5′-RACE with the use of two oligonucleotides complementary to the most 5′ end of the cDNAs (5′-AGCCACACCT-GAGCTCAAAGACCCACTTTG-3′ and 5′-GCCCC-ACCCAATTCTTTTGCCAAGAAGTAG-3′). The combined clones comprised the entire *A. thaliana* cDNA. The DNA sequence was determined for all fragments on both strands. The GenBank accession number is U40269.
28. A 446-bp partial cDNA from *C. elegans* encoding amino acids related to the *S. cerevisiae* Orc2p was identified in the NCBI dbEST database (no. 16625). A partial cDNA fragment was amplified by nested PCR with the use of DNA from a *C. elegans* λZAP cDNA library (gift of R. Barstead) and oligonucleotides complementary to the dbEST cDNA sequence (5′-ACGAGGGTGAAATTGATTGC-3′, 5′-TTGTGA-ATTGACGGCAAGAG-3′ and 5′-CTACAGTTGAT-CACATTTAC-3′). The amplified product was used as a probe to screen the λZAP cDNA library. A full-length cDNA was isolated and sequenced on both strands with the use of standard dideoxy sequencing. In addition, a genomic DNA fragment containing the partial cDNA sequence identified in the dbEST database was identified in the NCBI EMBL database (no. Z36949). Computer analysis of this DNA predicts an open reading frame encoding an Orc2-related protein consisting of nine exons spanning nucleotides 1596

to 5784. However, our results from the *CeORC2* cDNA sequence revealed that the authentic protein begins at position 2618 and the first exon ends at 2793 and excludes the first five predicted exons. Before termination at 5784, the DNA from 5781 to 5882 is spliced out and the last exon terminates at 5998. The GenBank accession number is U40270.

29. A 340-bp partial cDNA encoding amino acids related to *S. cerevisiae ORC2* was isolated by RT-PCR reaction using human HeLa cell mRNA. First-strand cDNA was synthesized from 2 μg of mRNA primed with oligo(dT). The resulting cDNA was used as a template for PCR with degenerate primers based on amino acids conserved among ScORC2, AtORC2, and CeORC2 (5′-[C/T]A[C/T]GGIGTIGGITCIAA[A/G][A/C]G-3′ and 5′-ATGTGGTCIA[C/T]IG[A/T]IGCIA[C/T]IA-3′). An amplified fragment was sequenced and found to be related to *S. cerevisiae ORC2*. This combination of primers also amplified a DNA fragment from *K. lactis* that was sequenced and found to be related to *S. cerevisiae ORC2* (*23*). The human DNA fragment was used as a probe to isolate a full-length human cDNA [see (*21*)]. Positive clones were plaque-purified and sequenced on both strands. The GenBank accession number is U40268.
30. M. Gossen, D. T. S. Pak, S. Hansen, J. Acharya, M. Botchan, *Science* **270**, 1674 (1995); A. E. Ehrenhofer-Murray, M. Gossen, D. T. S. Pak, M. R. Botchan, J. Rine, *ibid.*, p. 167.
31. pKG28 contains the full-length cDNA encoding HsORC1 under the control of the cytomegalovirus promoter fused to a single copy of the T7 epitope in pCGT [A. Wilson, M. G. Peterson, W. Herr, *Genes Dev.* **9**, 2445 (1995)]. Xba I and Bam HI sites were introduced into the cDNA encoding HsORC1 by PCR immediately upstream of the in-frame initiator methionine and immediately downstream of the stop codon, respectively. The entire coding region except for the 3′-most 120 nucleotides was replaced with a native DNA fragment, and sequencing of the 120 bp at the 3′ end revealed no mutations generated during PCR. Human 293 cells were transfected or mock-transfected with 10 μg of pKG28 by electroporation and were harvested 24 hours later. Whole-cell extracts were prepared and used for immunoprecipitation with rabbit antibody to amino acids 1 to 296 of HsOrc2p fused to GST. Immune complexes were collected on protein A–Sepharose (Pharmacia), washed, and separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose, and immunoblot analysis was done with a monoclonal antibody to the T7 epitope (Novagen). The ECL system was used for detection (Amersham).
32. A. Kornberg and T. A. Baker, *DNA Replication* (Freeman, New York, 1991), vol. 2.
33. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
34. We thank M. Hengartner, N. Kaplan, R. Martienssen, L. Rodgers, and J. Skowronski for providing various cDNA and genomic libraries; D. Casso and M. Caligiuri for *S. pombe* strains and vectors; A. Stenlund and M. Berg for help with transfections; M. Ockler for help with artwork; M. Weinreich and P. Kaufman for helpful discussions; and P. Kaufman for reading of the manuscript. Supported by the Japan Society for the Promotion of Science Postdoctoral Fellowship for Research Abroad to M.H. and by NIH grant CA13106 to B.S.

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Separation of Origin Recognition Complex Functions by Cross-Species Complementation

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Transcriptional silencing at the *HMR*a locus of *Saccharomyces cerevisiae* requires the function of the origin recognition complex (ORC), the replication initiator of yeast. Expression of a *Drosophila melanogaster Orc2* complementary DNA in the yeast *orc2-1* strain, which is defective for replication and silencing, complemented the silencing defect but not the replication defect; this result indicated that the replication and silencing functions of ORC were separable. The *orc2-1* mutation mapped to the region of greatest homology between the *Drosophila* and yeast proteins. The silent state mediated by *DmOrc2* was epigenetic; it was propagated during mitotic divisions in a relatively stable way, whereas the nonsilent state was metastable. In contrast, the silent state was erased during meiosis.

Position effects are influences on the expression of a gene as a function of its location within the genome. Classical position effects include the inactivation of X chromosomes in female mammals (*1*) and position effect variegation in *Drosophila* (*2*). The inheritance of position effects in dividing cells is epigenetic, that is, genetically identical cells can display different phenotypes. At present, the mechanism of establishing and maintaining epigenetic states of gene expression is unknown. In *S. cerevisiae*, a form of position effect occurs at the cryptic mating-type loci *HMR* and *HML* (*3*). These loci serve as donors of mating-type information in mating-type interconversion. Although the genes at *HMR* and *HML* are identical to the expressed sequences at the mating-type locus *MAT*, they are kept silent throughout the cell cycle. Silencing at *HMR* and *HML* requires the combined action of nearby regulatory sequences, called silencers, and multiple proteins, at least some of which function at the silencers. Interestingly, some silencers also act as chromosomal origins of replication (*4*). Altering the autonomously replicating sequence (ARS) consensus sites of the *HMR*-E silencer results in a loss of replication initiation at the silencer and a concomitant loss of silencing at *HMR* (*5*). Moreover, mutations in two subunits of ORC decrease silencing at *HMR* and reduce replication initiation at *HMR*-E (*6*, *7*). These observations have led to the hypothesis of some link between replication and silencing. We have challenged this hypothesis by investigating the cross-species complementation of ORC functions. If a homolog is capable of complementing both functions, the association between the two processes must be ancient and conserved. In contrast, if heterologous ORC proteins complement only the silencing function or only the replication function, then the two roles of ORC are experimentally separable and may have been separate in evolution.

To test the cross-species complementation of ORC functions, we determined whether the *Drosophila Orc2* gene (*DmOrc2*) (*8*) was able to complement the *orc2-1* mutation in the yeast ORC2 gene, which encodes the second largest subunit of ORC (*9*, *10*). A complementary DNA (cDNA) encoding DmOrc2 was placed under the control of the strong constitutive yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and the phosphoglycerate kinase terminator (PGK) (*11*). The recombinant gene was inserted into an integrative yeast–*Escherichia coli* shuttle vector and then integrated into the yeast genome (*12*). To test whether the protein encoded by *DmOrc2* was expressed in yeast, we prepared whole-cell extracts from strains that either did or did not contain the *DmOrc2* overexpression construct and analyzed them by immunoblotting with an antibody to DmOrc2 (Fig. 1). Immunoreactive DmOrc2 protein was present in the extracts from strains containing the GPD-*DmOrc2*-PGK hybrid gene, and it was not detected in strains lacking the construct. The electrophoretic mobility of the DmOrc2 protein expressed in yeast was consistent with its predicted molecular mass of 68.5 kD.

We next determined whether *DmOrc2* was capable of complementing the defects of the *orc2-1* mutation. By cloning and sequencing the *orc2-1* allele, we found that this mutation resulted in a change of a proline to a leucine residue at position 603 of the Orc2 protein, a residue that was conserved between *Drosophila* and yeast. The *orc2-1* mutation affects both the replication initiation function and the silencing function of the Orc2 protein (*6*). The rep-

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lication defect causes a temperature-sensitive cell-cycle defect, presumably because essential parts of the genome fail to be replicated at the nonpermissive temperature. Moreover, *orc2-1* strains exhibit reduced replication initiation at chromosomal origins, and they have a defect in the maintenance of both centromere-based and 2μ plasmids, even at the permissive temperature (6). The silencing defect of *orc2-1* is most evident at a sensitized *HMR***a** allele (*HMR-SSΔI*) (6). In a *MAT*α strain, this defect leads to the simultaneous expression of **a** and α information, which produces a nonmating phenotype. This loss of silencing at *HMR***a** correlates with a drastically reduced ability of the *HMR*-E silencer to act as a chromosomal origin of replication.

To assay the complementation of the *orc2-1* mutation by *DmOrc2*, we introduced the GPD-*DmOrc2*-PGK vector into the *orc2-1* strain and compared the temperature sensitivity and mating ability of these transformants to that of the *orc2-1* strain (Fig. 2). Two types of *DmOrc2*-expressing *orc2-1* transformants were recovered. In some transformants, the mating ability was restored to nearly that of a wild-type strain, indicating that *DmOrc2* was able to restore silencing at *HMR***a** in these strains (JRY5213; Fig. 2C, c). In other transformants, the mating ability was not restored (JRY5212; Fig. 2C, d), indicating that *DmOrc2* was unable to restore silencing of *HMR***a** in these strains. However, neither of the two types of transformants was able to grow at the nonpermissive temperature (Fig. 2B); this result indicated that *DmOrc2* could not provide the essential replication function that is missing in an *orc2-1* mutant. Therefore, *DmOrc2* was capable of complementing the silencing defect, but not the replication defect, of *orc2-1*. The sequence homology between the *Drosophila* and yeast Orc2 proteins thus reflected a functional homology. Moreover, this result suggested that the roles of ORC in silencing and in replication could be separated.

We performed several additional tests to determine whether DmOrc2 was providing any replication function in yeast: (i) *DmOrc2*-expressing *orc2-1* strains had no detectable growth advantage over *orc2-1* strains at the permissive temperature or at any semipermissive temperature tested, regardless of whether the strains were repressed or derepressed at *HMR* (*13*). (ii) *DmOrc2* was unable to relieve the plasmid maintenance defect of *orc2-1* (*14*). (iii) *DmOrc2* was unable to rescue the inviability of a strain containing an *orc2* null allele (*15*). In principle, *DmOrc2* could have restored silencing at *HMR* by restoring replication initiation at *HMR*-E in *orc2-1* strains without restoring sufficient replication function to allow viability. To test this possibility, we performed two-dimensional origin-mapping assays (Fig. 3). As observed earlier (6), even at the permissive temperature, the *orc2-1* mutation caused a decrease in replication initiation at the *HMR*-E silencer relative to that of a wild-type *ORC2* strain (Fig. 3, A and B). However, we were unable to detect any appreciable increase in replication initiation at the silencer in *orc2-1* strains containing the GPD-*DmOrc2*-PGK hybrid gene (Fig. 3C). As a second measure of the silencer origin efficiency, we determined the loss rate of plasmids that carried *HMR*-E as the sole origin. The presence of *DmOrc2* was unable to stabilize the extreme plasmid loss in *orc2-1* strains (*14*). Thus, these findings provided genetic and physical evidence that the DmOrc2 protein did not support replication initiation in yeast.

It was surprising that *DmOrc2* complemented the silencing defect of *orc2-1* in some transformants, but not in others (Fig. 2). These strains were genetically identical, and yet they displayed different phenotypes. Therefore, this complementation had an epigenetic component, which prompted further investigation. Testing whether the two different classes of *DmOrc2* transformants had different amounts of expression of DmOrc2 (Fig. 1, lanes 2 and 3) revealed no difference in the amounts of DmOrc2 expressed in strains in which *HMR***a** was or was not silenced. Thus, differences in *DmOrc2* expression were not the source of the variation. Chromosomal replication initiation at the *HMR*-E silencer was also compared in the two different cell types

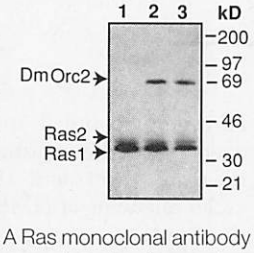

Fig. 1. Detection of DmOrc2 expressed in yeast. Cell extracts of the *orc2-1* strain JRY4475 (lane 1) and the *DmOrc2*-expressing strains JRY5212 (lane 2) and JRY5213 (lane 3) were analyzed for the presence of DmOrc2. JRY5212 and JRY5213, which are transformants of JRY4475 with pJR1722, contained a derepressed and a repressed *HMR***a** allele, respectively. The strains were grown to mid-log phase, and whole cell lysates were prepared by glass bead lysis [(*22*), except that phosphate-buffered saline containing protease inhibitors was used as the lysis buffer]. The protein equivalent of 0.5 OD_{600} units of cells was separated on an 8% SDS–polyacrylamide gel and immunoblotted with a polyclonal antibody to DmOrc2 (*8*). A Ras monoclonal antibody (Y13-259, Oncogene Science), which recognizes both Ras1p and Ras2p, was used as a loading control. Immunocomplexes were visualized with the enhanced chemiluminescence system ECL (Amersham).

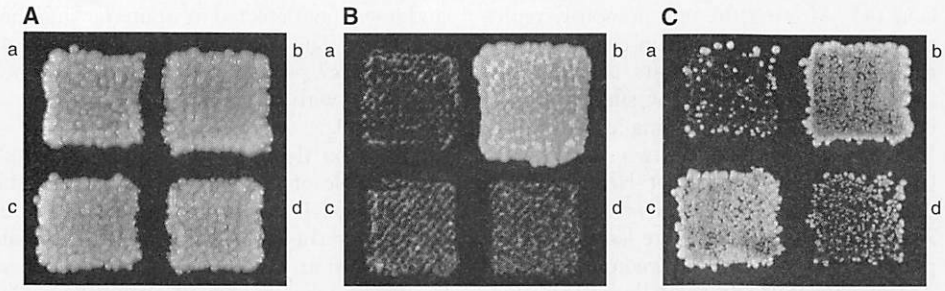

Fig. 2. *DmOrc2* complemented the silencing defect, but not the temperature-sensitive growth defect, of the yeast *orc2-1* mutation. The *MAT*α *HMR-SSΔI* strains *orc2-1* (JRY4475) [(a) in each panel], *ORC2* (JRY4473) [(b) in each panel], and two *DmOrc2*-expressing *orc2-1* strains (JRY5213 and JRY5212, respectively) [(c) and (d) in each panel] were assayed for their growth at the permissive temperature (23°C) (**A**) and at the restrictive temperature (37°C) (**B**), as well as for their ability to mate with a *MAT***a** *his4* tester strain (JRY2726) at the permissive temperature (**C**).

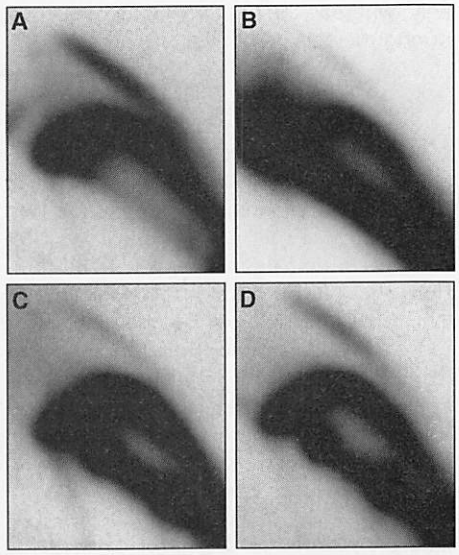

Fig. 3. *DmOrc2* did not increase replication initiation at the *HMR*-E silencer in *orc2-1* strains. A Hind III–Bgl II *HMR* fragment was analyzed for the presence of replication intermediates with the use of two-dimensional origin-mapping gels (*23*), as described (*6*). The *MAT*α *HMR-SSΔI* strains were (**A**) a wild-type *ORC2* strain (JRY4473), (**B**) an *orc2-1* strain (JRY4475), (**C**) a *DmOrc2*-expressing *orc2-1* strain with a repressed *HMR* locus (JRY5213), and (**D**) a *DmOrc2*-expressing *orc2-1* strain with a derepressed *HMR* locus (JRY5212).

(Fig. 3, C and D). However, regardless of the amount of silencing at *HMR***a** in these strains, no difference in replication initiation from the silencer was detected in either cell type.

Because individual colonies could be isolated that were either silenced or not silenced at *HMR*, both of these states were stable enough to form a colony of a single type. However, from colonies of either type, we were able to recover clonal isolates with the characteristics of the opposite type. For example, a transformant colony in which *HMR***a** was silenced contained a subset of cells in which *HMR***a** was not silenced. Conversely, a transformant colony in which *HMR***a** was not silenced also harbored cells in which *HMR***a** was silenced. Prolonged propagation of a strain in which *HMR***a** was not silenced led to an increase in the percentage of cells with a silenced *HMR* locus (Fig. 4). This increase in silencing was *DmOrc2*-dependent, because a similar increase was not observed with the *orc2-1* strain alone. The accumulation of clones with a silenced *HMR* was not the result of a selective growth advantage, because strains with repressed or derepressed *HMR* had the same growth rates. Moreover, the ratio of cells with silenced versus nonsilenced *HMR* loci in a transformant colony that was predominantly silenced at *HMR* varied only slightly during mitotic growth. These results indicated that the *DmOrc2*-dependent silent state was more stably propagated in mitosis than was the nonsilent state. The nonsilent state was metastable in the presence of *DmOrc2*, such that the fraction of cells silenced at *HMR* increased over time. These data were consistent with a role of the DmOrc2 protein in the establishment of the silent state, the maintenance of the silent state, or both.

The persistence of the silent state at *HMR* mediated by *DmOrc2* was tested during meiosis. For this purpose, strains with either a repressed or a derepressed *HMR* locus were mated to an isogenic wild-type strain, and the mating phenotype of the *orc2-1* progeny containing *DmOrc2* was evaluated. In a cross in which the *DmOrc2*-containing parent harbored a derepressed *HMR* locus, the majority of the *orc2-1* offspring carrying *DmOrc2* also were derepressed at *HMR* (33 of 41). A cross with a parent containing a repressed *HMR* locus also yielded a majority of progeny in which *HMR***a** was derepressed (40 of 45). Therefore, the *DmOrc2*-dependent silent phenotype was much less stable during meiosis than it was during mitosis. These observations indicated an erasure of the silent phenotype during meiosis, and thus they suggest that mitotic inheritance is mechanistically distinct from meiotic inheritance.

The ability of *DmOrc2* to complement the silencing defect provides the first evidence that the structural similarity between the *Drosophila* and yeast proteins reflects a functional homology. However, as measured by genetic and physical methods, *DmOrc2* was unable to promote replication in yeast. Nonetheless, Gossen *et al.* and Gavin *et al.* (*8*, *16*) strongly suggest that *DmOrc2* does play a role in replication in *Drosophila* because of the highly conserved structure of the ORC complex and the existence of homologs in a variety of eukaryotes. The inability of *DmOrc2* to complement the replication defect in yeast could be explained in several ways. Perhaps a yeast ORC complex containing DmOrc2 was unable to recognize enough yeast origins of replication to replicate the genome. Alternatively, the DmOrc2-containing complex may be unable to interact with other key replication proteins, such as Cdc7p (*17*) or Cdc6p (*18*).

The apparent lack of replication function provided by *DmOrc2* in yeast indicated that repression at the *HMR* locus could be restored independently of replication initiation at the *HMR*-E silencer. Thus, *DmOrc2* can be considered an *orc2* allele that separates the replication and silencing functions of ORC. The ability of *DmOrc2* to provide the silencing function indicates that ORC complexes containing DmOrc2 are able to communicate with other silencing proteins. Could these data reflect a role of ORC in silencing in *Drosophila*? It is possible that ORC plays no role in silencing in *Drosophila*; the ability of *DmOrc2* to restore silencing in yeast would then merely reflect the role of the yeast ORC complex in silencing and the ability of *DmOrc2* to stabilize or restore the function of the complex in *orc2-1* mutants. Alternatively, at face value, the data are also compatible with a role for *Drosophila* ORC in the control of gene expression in *Drosophila*. This model would imply an interaction between *Drosophila* ORC and regulatory proteins in *Drosophila* that would be equivalent to an interaction between yeast ORC and silencing proteins. However, if *Drosophila* ORC is involved in controlling gene expression, there must be at least some differences in the silencing mechanism between yeast and other eukaryotes, because a portion of Orc1p that is required for silencing in *Saccharomyces* is not conserved in other eukaryotes (*16*).

The epigenetic component of *DmOrc2*'s silencing complementation in yeast bears similarities to the variegated position effects in *Drosophila* (*19*). Therefore, it will be of interest to learn whether *Drosophila* ORC plays a role in these position effects. The epigenetic behavior of silencing conferred by *DmOrc2* might also reflect a decreased ability of the DmOrc2-containing complex to interact with the yeast silencing proteins, in particular with the Sir1 protein (*20*). The erasure of the silent phenotype in cells during meiosis suggests that such a multiprotein complex dissociates during meiosis or within a few mitotic divisions immediately after meiosis. The epigenetic inheritance of silencing is not restricted to the silencing brought about by *DmOrc2*, because *sir1* mutants behave in a similar way. On the basis of these parallels, we predict an intimate relation between ORC and the Sir1 protein in silencing.

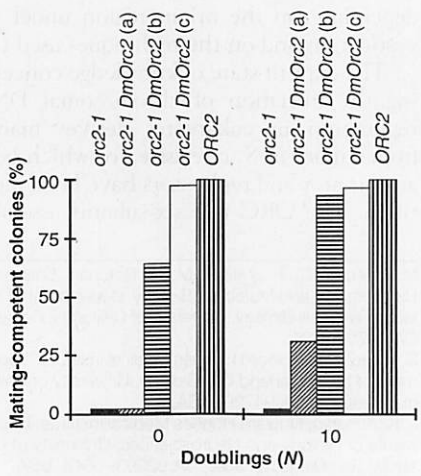

Fig. 4. *DmOrc2* increased the percentage of *orc2-1* clones with a repressed *HMR* locus. Three independent *MATα HMR-SSΔI DmOrc2 orc2-1* strains that initially contained varying amounts of derepressed *HMR* loci were analyzed for changes in the number of clones within the culture that were repressed at *HMR*. The *DmOrc2* strains initially contained *HMR* loci that were (a) predominantly derepressed, (b) 65% repressed, or (c) predominantly repressed. The strains were grown in liquid culture during 10 doubling times, samples were plated on complete medium, and the repression state at *HMR* of individual colonies within the culture was tested by determining their mating ability. The colonies were rated either as mating-competent (*HMR* repressed) or as mating-incompetent (*HMR* derepressed), and this rating is given as the percentage of mating-competent colonies among all colonies tested. The sample sizes were 120 to 160 individual colonies per strain and time point. The *orc2-1* strain JRY4475 and the *ORC2* strain JRY4473 served as controls.

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12. The coding sequence for *DmOrc2* was excised as a 1.9-kb Bsp HI–Sal I fragment from a pNB40-based plasmid [N. H. Brown and F. C. Kafatos, *J. Mol. Biol.* **203**, 425 (1988)] containing the cDNA (*8*), treated with Klenow polymerase and inserted into the Sma I site of pG-3 (*11*) such that the *DmOrc2* transcription start was next to the GPD promoter. This GPD-*DmOrc2*-PGK expression construct was then inserted as a Hind III–Xba I cassette into YIplac128 (*21*) to create pJR1722. The yeast strain JRY4475 (*MAT*α, *HMR-SSΔI, ade2-1, his3-11,15, trp1-1, leu2-3,112, ura3-1, can1-100, orc2-1*) was transformed to leucine prototrophy with Eco RV–linearized pJR1722 [R. J. Klebe, J. V. Harriss, Z. D. Sharp, M. G. Douglas, *Gene* **25**, 333 (1983)].
13. The growth of the *DmOrc2*-expressing *orc2-1* strains JRY5212 and JRY5213 was compared to that of JRY4475 (*orc2-1*) and, as a reference, to the growth of the isogenic *ORC2* strain JRY4473. The strains were streaked on complete medium and incubated at 23°, 26°, 30°, 34°, and 37°C for 3 days, and their growth rates in liquid culture at 23°C were compared.
14. The loss rates (*5*) for plasmids with ARS1 (pJR1469), ARSH4 (pRS316), or the synthetic *HMR*-E silencer (pJR950) as the sole origins of replication were determined in an *ORC2* strain (JRY3009), in an *orc2-1* strain (JRY4475), and in two *DmOrc2*-expressing *orc2-1* strains (JRY5212 and JRY5213) (*5*). The loss rates, expressed as plasmid loss events per cell division, of plasmids replicated by ARS1 or ARSH4 were low (<0.001) in the wild-type strain but were increased in the *orc2-1* strains (0.060 and 0.155 for ARS1 and ARSH4, respectively). The *orc2-1* mutation increased the loss rate for plasmids replicated by the synthetic silencer from 0.024 in the *ORC2* strain to 0.190. The loss rates for *DmOrc2*-expressing *orc2-1* strains were 0.039 ± 0.025, 0.146 ± 0.022, and 0.197 ± 0.069 for ARS1, ARSH4, and the synthetic silencer, respectively.
15. A null allele of *ORC2* was constructed by replacing an internal Msc I fragment of a 5.5-kb Sac I–Sal I *ORC2* fragment by the 1.8-kb Bam HI fragment of *HIS3*. In that *ORC2* is an essential gene, a diploid heterozygous for *orc2-Δ2::HIS3* was constructed by transforming the wild-type diploid JRY4014 to histidine prototrophy with the disruption construct. JRY4014 is a W303-based diploid strain heterozygous for the *HMR-SSΔI* allele. An *ORC2/orc2-Δ2::HIS3* diploid was then transformed with a *URA3*-marked yeast–*E. coli* shuttle vector carrying *ORC2*, pJR1263 (*9*). Tetrads were dissected from the transformed diploid, and a haploid *MAT*α *HMR-SSΔI orc2-Δ2::HIS3* strain containing pJR1263 (JRY5010) was recovered. JRY5010 was transformed to leucine prototrophy with YCplac111 (*21*) containing the GPD-*DmOrc2*-PGK cassette (pJR1720). Several transformants were then tested for their ability to lose the *URA3-ORC2* plasmid by assaying their resistance to 5-fluoroorotic acid (FOA) [J. D. Boeke, F. LaCroute, G. R. Fink, *Mol. Gen. Genet.* **197**, 345 (1984)]. In contrast to JRY5010 cells transformed with a *LEU2-ORC2* plasmid, JRY5010 cells transformed with pJR1720 were unable to grow on FOA, which indicated that they could not lose the *URA3-ORC2* plasmid. Thus, *DmOrc2* expression was unable to rescue the inviability of the *orc2* deletion.
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A *Drosophila* Homolog of the Yeast Origin Recognition Complex

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Genes from *Drosophila melanogaster* have been identified that encode proteins homologous to Orc2p and Orc5p of the *Saccharomyces cerevisiae* origin recognition complex (ORC). The abundance of the *Drosophila* Orc2p homolog DmORC2 is developmentally regulated and is greatest during the earliest stages of embryogenesis, concomitant with the highest rate of DNA replication. Fractionation of embryo nuclear extracts revealed that DmORC2 is found in a tightly associated complex with five additional polypeptides, much like the yeast ORC. These studies will enable direct testing of the initiator-based model of replication in a metazoan.

DNA replication in higher eukaryotes is intricately regulated, both temporally and spatially, within each cell cycle and throughout development. The mechanisms underlying this regulation remain largely obscure, primarily because the sites and proteins involved in initiation have not been clearly identified. In the classic replicon model (*1*), a positive-acting factor (the initiator) acts at a specific DNA sequence (the replicator) to direct DNA synthesis to a nearby start site, the origin of replication. This concept has proven to be valid for prokaryotes as well as for eukaryotic DNA viruses (*2*). However, metazoan initiators that recognize chromosomal DNA have not been described. Moreover, the exact nature of the metazoan replicon is subject to controversy; start sites of DNA replication have been mapped to relatively short DNA sequences or to widespread initiation zones, depending on the origin region under investigation and on the techniques used (*3*).

The current state of knowledge concerning the initiation of chromosomal DNA replication in eukaryotes derives mainly from studies in *S. cerevisiae*, for which both an initiator and replicators have been identified. The ORC is a six-subunit assembly that binds in a site-specific, adenosine triphosphate (ATP)–dependent manner to autonomously replicating sequence (ARS) consensus sites, which define budding yeast origins of DNA replication (*4*). Despite the large number of replicator sites in a yeast chromosome and the sequence variations within these sites, it appears as if one vital initiator complex is central to the orchestration of the events that lead to DNA synthesis. The ORC influences the rate of firing at all active origins that have been examined (*5*) and also participates in transcriptional silencing at ARS elements of the yeast mating type loci (*6*, *7*). Three ORC subunits (Orc2p, Orc5p, and Orc6p) have been described; they have been shown to be essential for viability and, in the case of Orc2p and Orc5p, for plasmid maintenance as well (*6*, *8*, *9*).

A genomic DNA sequence in the region of the *Drosophila* genome proximal to the *inositol polyphosphate-1-phosphatase* gene (*IPP*) showed homology to *S. cerevisiae* ORC2 (*10*). We used the initially defined region of sequence homology to generate hybridization probes, and we identified complementary DNA (cDNA) clones in two independent early embryonic libraries (*11*). The sequences of these cDNAs predicted a protein that showed ~30% amino acid identity and ~56% homology to the COOH-terminus (residues 430 to 547) of *S. cerevisiae* Orc2p (Fig. 1B). Because the homology was less pronounced at the NH_2-termini of the two proteins, the average amino acid identity was 21% (37% homology).

Antibodies to this *Drosophila* Orc2p homolog, DmORC2 (*12*), were used to analyze protein extracts from *Drosophila* embryos col-

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lected at various time points during development. DmORC2 was most abundant in embryos 0 to 4 hours old (Fig. 2) and constituted ~0.01% of total protein at this stage. Fractionation of embryos into nuclear and cytosolic extracts (*13*) and protein immunoblotting of equal amounts of protein from these extracts with antibodies to DmORC2 (anti-DmORC2) showed that DmORC2 was localized predominantly in the nucleus. Accordingly, we processed embryo nuclear extracts by conventional chromatography to determine whether DmORC2 was part of a multiprotein complex, as would be expected if the eukaryotic DNA replication initiation machinery is conserved.

Nuclear extracts (*13*) were first fractionated on a heparin-POROS (Perseptive Biosystems) matrix, and the elution of DmORC2 was followed by protein immunoblot analysis with affinity-purified anti-DmORC2 (*12*). The eluate that contained DmORC2 was pooled and further fractionated (Fig. 3A). About half of the DmORC2 separated on the Sephacryl-300 (Pharmacia) step into a high-molecular-mass fraction estimated to be >500 kD, whereas the remainder appeared to be in complexes of lower molecular mass. We continued to analyze the composition of the higher molecular mass complex. Ion-exchange chromatography with Mono-Q (Pharmacia) (Fig. 3A) showed that DmORC2 eluted in a sharp peak, which implied a homogeneous physical interaction with the matrix.

Immunoprecipitation of DmORC2 from the Mono-Q peak fractions and analysis of the immunoprecipitates by SDS–polyacrylamide gel electrophoresis (PAGE) revealed that DmORC2 was consistently associated with five other proteins (Fig 3B). This complex of six polypeptides was also observed after the complex was washed in buffer containing 1 M KCl or 1 M urea (*12*). The molecular mass of each polypeptide was inferred by the electrophoretic mobility of the protein bands relative to silver-stained size standards in several independent immunoprecipitation experiments (Fig. 3B). The sum of these molecular masses and the apparent stoichiometry of the subunits yielded a predicted molecular mass of 395 kD for the *Drosophila* complex; this value is close to the 413 kD estimated by similar means for the yeast complex.

The Mono-Q material was further fractionated on a Mono-S column, from which the DmORC2-containing peak eluate was analyzed by glycerol gradient centrifugation (Fig. 3C); the results again demonstrated that DmORC2 is contained in a complex. The sedimentation data revealed an apparent molecular mass of ~235 kD for the DmORC2 complex (comigrating with the catalase marker), as detected by protein immunoblotting with anti-DmORC2. This peak coincided with the peak of the five other *Drosophila* proteins, as judged by silver staining of the glycerol gradient fractions containing DmORC2 (Fig. 3C). Yeast ORC also has an apparent sedimentation coefficient close to that of catalase (*4*). Recombinant DmORC2 (*12*), in contrast, sedimented as a sharp peak at an apparent molecular mass of ~100 kD. We concluded from these data that the complex characterized here had properties that were similar to those of ORC purified from budding yeast with respect to the number of subunits, combined molecular mass, and sedimentation behavior. Thus, it was likely that the two complexes had homologous structures.

Given these similarities between the *Drosophila* and yeast ORCs, we anticipated that the DmORC2-associated proteins would have homology to other yeast ORC subunits. We cloned a gene with significant sequence homology to *S. cerevisiae ORC5* on the basis of sequence information released from the *Drosophila* Genome Project (*14*). Complementary DNAs of the putative *DmORC5*, isolated from the embryonic library that contained full-length *DmORC2* cDNAs (*11*), revealed an open reading frame (ORF) with

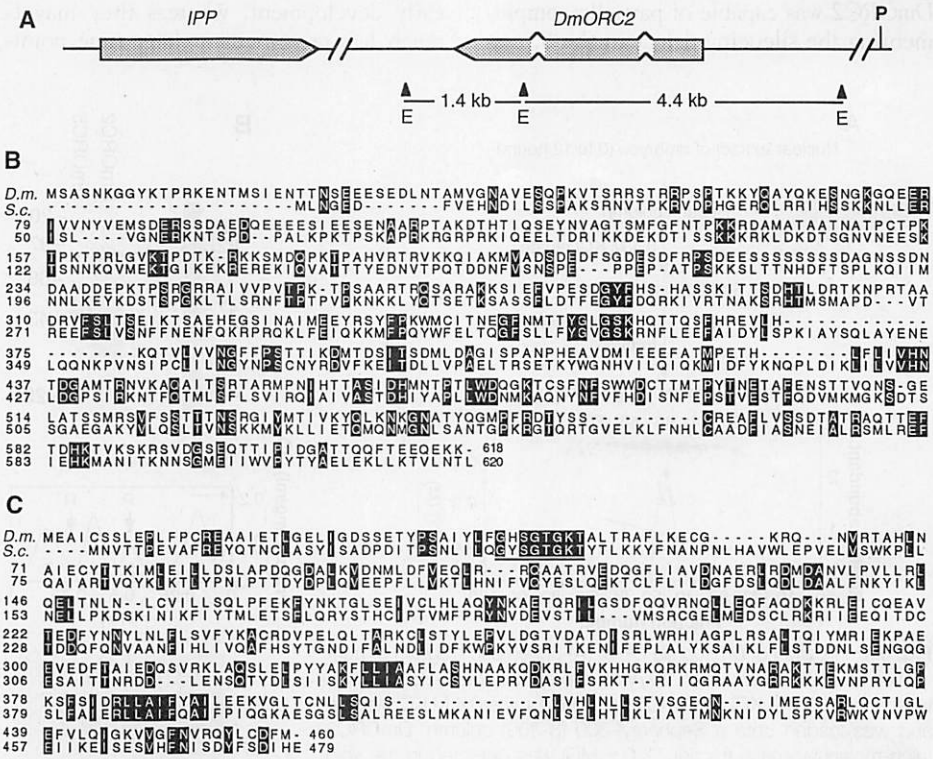

Fig. 1. *Drosophila* homologs of *S. cerevisiae* ORC proteins. (**A**) Schematic presentation of the genomic region of the *Drosophila DmORC2* gene. The ORFs of the *IPP* and *DmORC2* genes are indicated by arrowheads. Featured in the map are the Eco RI restriction sites (E) used for the initial subcloning of *DmORC2*, the position of the P-element insertion (P) in the particular *Drosophila* strain in which *DmORC2* was initially found, and the positions of the two introns (nonshaded areas) in the genomic *DmORC2* DNA. (**B**) The deduced amino acid sequence encoded by the *DmORC2* gene is shown in alignment (*22*) with *S. cerevisiae* Orc2p. Identical residues are highlighted. (**C**) The deduced amino acid sequence encoded by the *DmORC5* gene is shown in alignment with *S. cerevisiae* Orc5p. The putative nucleotide binding site is underlined. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

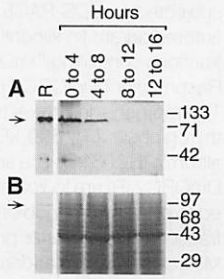

Fig. 2. Abundance of DmORC2 in early stages of *Drosophila* development. (**A**) Protein immunoblot analysis of total embryo extracts at various developmental stages. Collected embryos were subjected to Dounce homogenization in SDS sample buffer. Extracts were fractionated by SDS-PAGE and transferred to a nylon membrane. Protein was incubated with DmORC2-specific rabbit antiserum (*12*) and visualized by chemiluminescence with horseradish peroxidase–coupled protein A and ECL reagents (Amersham). (**B**) A Coomassie blue–stained gel of the same material used in (A) to verify that equivalent amounts of total protein were analyzed for DmORC2 at the different stages. Half the amount of cell extract was loaded per lane relative to (A). Recombinant DmORC2 (R) was used as a standard [10 ng in (A), 150 ng in (B)]. The positions and sizes (in kilodaltons) of the molecular mass markers are indicated at the right.

22% identity and 39% homology to yeast Orc5p (Fig. 1C). The predicted NH_2-terminus of DmORC5 contained a purine nucleotide binding site P-loop (*15*), as does yeast Orc5p (*8*). However, the utility of this putative ATP binding site in yeast is unclear (*8*). The in vitro translated product from the *DmORC5* cDNA comigrated in SDS-PAGE with the fourth largest subunit of the *Drosophila* ORC (Fig. 3B). The identity of this subunit with the predicted gene product of *DmORC5* was confirmed by peptide sequence analysis (*16*).

Taken together, our data suggest that this *Drosophila* complex is a functional homolog of the yeast initiator ORC. Indeed, *DmORC2* was capable of partially complementing the silencing defect in the *S. cerevisiae* temperature-sensitive *orc2-1* mutant, which demonstrated functional conservation between these two genes (*17*). Additionally, the distribution of DmORC2 during embryogenesis (Fig. 2) correlated with the observation that major waves of rapid cell division occur in embryos during the first 4 hours of development and then taper off (*18*). In these rapid nuclear cleavages, the requisite high rate of DNA synthesis is achieved, at least in part, by a frequency of detectable active origins that is 100 times the frequency seen in cultured *Drosophila* cell lines (*19*). Initiator proteins that determine origins of replication in *Drosophila* are therefore expected to be abundant during early development, whereas they may be much less prominent at later time points, when there are fewer active origins.

If DmORC proves to be a bona fide initiator, we would expect to find its cognate replicator sequence. The interaction between these two components in *Drosophila* may, however, be subject to temporal and spatial control during development. In view of this potential complexity, the accessibility of *Drosophila* extracts to biochemical analysis (*20*) should facilitate the identification of replicators in a metazoan and of the mechanisms that underlie initiation of DNA replication at these loci.

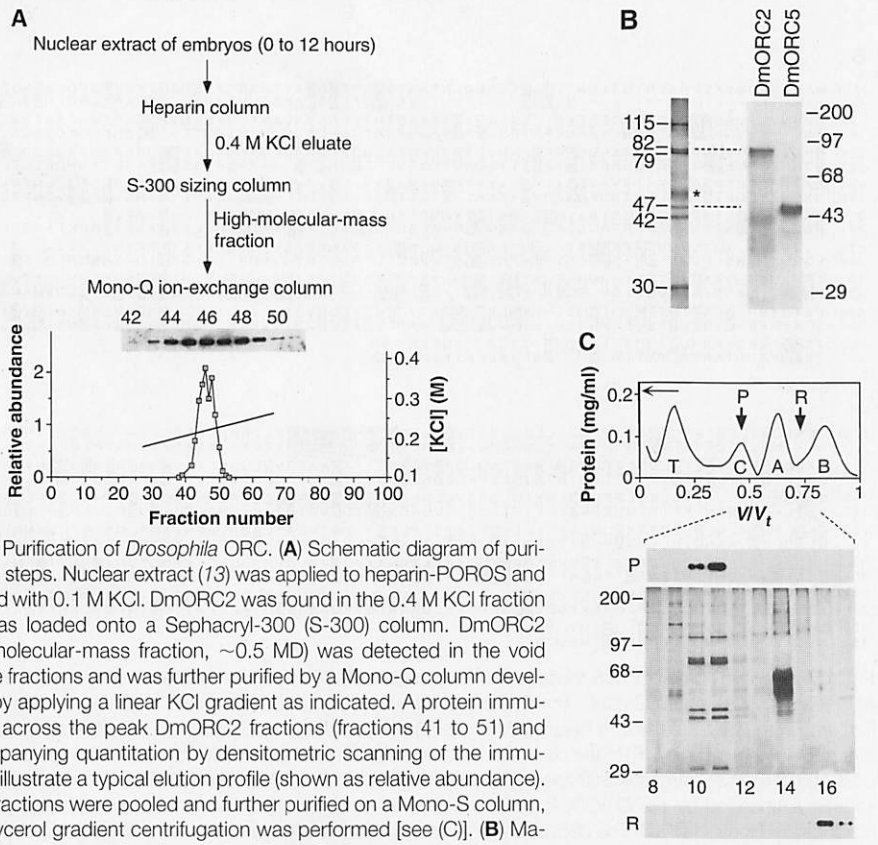

Fig. 3. Purification of *Drosophila* ORC. (**A**) Schematic diagram of purification steps. Nuclear extract (*13*) was applied to heparin-POROS and washed with 0.1 M KCl. DmORC2 was found in the 0.4 M KCl fraction and was loaded onto a Sephacryl-300 (S-300) column. DmORC2 (high-molecular-mass fraction, ~0.5 MD) was detected in the void volume fractions and was further purified by a Mono-Q column developed by applying a linear KCl gradient as indicated. A protein immunoblot across the peak DmORC2 fractions (fractions 41 to 51) and accompanying quantitation by densitometric scanning of the immunoblot illustrate a typical elution profile (shown as relative abundance). Peak fractions were pooled and further purified on a Mono-S column, and glycerol gradient centrifugation was performed [see (C)]. (**B**) Material from the peak fraction of the Mono-Q eluate (fraction 46) was immunoprecipitated with affinity-purified anti-DmORC2 (*12*). Precipitates were analyzed by SDS-PAGE and stained with silver (left panel). The asterisk indicates the position of the immunoglobulin G heavy chain. In the right panel, coupled in vitro transcription and translation extracts (Promega) were programmed with T3 promoter–driven *DmORC2* or *DmORC5* cDNAs. Translation products were labeled with [^{35}S]methionine, subjected to SDS-PAGE, dried, and analyzed by autoradiography. The molecular mass markers for the autoradiogram (in kilodaltons) are indicated. (**C**) Glycerol gradient sedimentation of DmORC. Mono-S peak fractions containing DmORC2 were centrifuged in a 15 to 35% glycerol gradient with 0.1 M KCl-HEMG (*13*). Recombinant DmORC2 protein and molecular mass standards were sedimented in parallel gradients (*23*). The top panel indicates the profile of the molecular mass markers as assayed by the method of Bradford: thyroglobulin (T), 669 kD; catalase (C), 232 kD; alcohol dehydrogenase (A), 150 kD; and bovine serum albumin (B), 66 kD. The sedimentation positions of the peak fraction for the complex (P) and the recombinant DmORC2 (R) are indicated. In panel P, glycerol gradient fractions containing Mono-S material were subjected to protein immunoblotting with anti-DmORC2. The panel below shows the same fractions (sedimentation fractions 8 to 17) after precipitation by trichloroacetic acid and analysis by SDS-PAGE and silver staining. Molecular mass standards are indicated. In panel R, glycerol gradient fractions containing recombinant DmORC2 were subjected to protein immunoblotting with anti-DmORC2, and the results were aligned with respect to the lanes above.

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10. The *Drosophila* genomic DNA that potentially encodes a protein with sequence similarity to yeast Orc2p was identified in the course of analyzing the nearby *IPP* gene. A P-element insertion in close proximity to the *IPP* gene was rescued, and the genomic DNA was further subcloned as Eco RI fragments. The homology to yeast Orc2p was identified by sequencing the insert boundaries of two of these subclones, which were later identified as sequences flanking the Eco RI site indicated within the *DmORC2* ORF (Fig. 1A).
11. A plasmid cDNA library from 4- to 8-hour *Drosophila* embryos [N. H. Brown and F. C. Kafatos, *J. Mol. Biol.* **203**, 425 (1988)] was transformed into *Escherichia coli* SCS-1 (Stratagene); 1.4×10^6 transformants were obtained and were transferred at high density to nylon membranes. These membranes were screened [D. Hanahan and M. Meselson, *Recombinant DNA Methodology* (Academic Press, London, 1989), pp. 267–275] with ^{32}P-labeled DNA fragments from the *Drosophila* genomic subclones (*10*) that encompassed the sequence homology to the yeast *ORC2* gene. About 80 positive clones could be identified in the initial hybridization, and eight were isolated. Restriction enzyme analysis indicated that they contained a common cDNA sequence. The nucleotide sequence of the longest cDNA isolated (clone 2-2) revealed an ORF (Fig. 1B). In parallel, a λgt10 cDNA library from 3- to 12-hour *Drosophila* embryos, which contained 5×10^5 independent clones, was used to infect BNN102 hosts, and 3×10^5 plaques were screened with the same hybridization probes as described above. Thirty clones remained after three rounds of plaque purification, of which 10 were characterized. All phage clones contained sequences that matched the ORF of clone 2-2.
12. *DmORC2* was cloned into the T7 expression vector RSET-A (Invitrogen) fused to an epitope encoding six histidines. The fusion construct was transformed into *E. coli* BL21 LysS (Novagen). Expression of His-tagged DmORC2 was induced with isopropyl-β-D-thiogalactopyranoside, and the protein was purified by nickel-chelate chromatography according to standard procedures. For expression of the protein in Sf9 cells, *DmORC2* was inserted into a BaculoGold vector (Pharmingen) and expressed with a polyoma middle-T antigen epitope tag. The baculovirus-expressed DmORC2 was purified by immunoaffinity chromatography with the use of protein G–Sepharose (Pharmacia) conjugated to monoclonal antibodies against the polyoma epitope tag.

Polyclonal rabbit antibodies were obtained after three or four immunizations with both *E. coli*– and baculovirus-expressed DmORC2. These polyclonal antibodies were affinity-purified (*21*) by incubation with baculovirus-produced DmORC2 protein coupled to Reactigel beads (Pierce). The eluted antibodies were bound to protein A–Sepharose (Pharmacia) and chemically cross-linked to this support with dimethylpimelimidate (*21*). For immunoprecipitation, 1 μl of these beads was added directly to the respective chromatographic or glycerol gradient fraction, adjusted to 100 mM KCl, incubated for 2 hours at 4°C, and washed six times with 0.1 M KCl–HEMG [25 mM Hepes (pH 7.6), 0.5 mM EDTA, 12.5 mM $MgCl_2$, and 10% glycerol] (*14*). The recovered material was resuspended directly in SDS sample buffer and analyzed by SDS-PAGE.

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23. Fraction number variation among different glycerol gradients (23 fractions for the molecular mass standards, 20 fractions for recombinant DmORC2, and 21 fractions for the complex) was corrected by normalization of each fraction to V/V_t (the ratio of cumulative eluted volume to total gradient volume). The leftmost and rightmost lanes of each protein immunoblot and silver stain correspond to fractions eluted at 36% and 79% of the total volume, respectively.
24. We thank C. Zuker for providing the *DmORC2* genomic clone, D. Rio for bringing the initial finding of the Zuker lab to our attention, M. Levine (University of California, San Diego) for the plasmid cDNA library, T. Kornberg for the λgt10 cDNA library, D. Rio and R. Tjian for encouragement and technical advice, and A. Ho for help. Supported by a pilot project grant from the National Institute of Environmental Health Sciences Mutagenesis Center (ES-01896), National Cancer Institute grant CA30490, and a Human Frontier Science Program Long-Term Research Fellowship (M.G.). Sequences of the *DmORC2* and *DmORC5* cDNAs have been deposited in GenBank (accession numbers pending).

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Early-Onset Epilepsy and Postnatal Lethality Associated with an Editing-Deficient *GluR-B* Allele in Mice

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The arginine residue at position 586 of the GluR-B subunit renders heteromeric α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)–sensitive glutamate receptor channels impermeable to calcium. The codon for this arginine is introduced at the precursor messenger RNA (pre-mRNA) stage by site-selective adenosine editing of a glutamine codon. Heterozygous mice engineered by gene targeting to harbor an editing-incompetent *GluR-B* allele synthesized unedited GluR-B subunits and, in principal neurons and interneurons, expressed AMPA receptors with increased calcium permeability. These mice developed seizures and died by 3 weeks of age, showing that GluR-B pre-mRNA editing is essential for brain function.

Glutamate receptors sensitive to AMPA are ligand-activated cation channels that mediate the fast component of excitatory postsynaptic currents in central neurons (*1*). These channels are assembled from four related subunits (GluR-A to GluR-D, or GluR1 to GluR4) (*2*), with the GluR-B subunit rendering the channel almost impermeable to Ca^{2+} (*3*). The molecular determinant for this dominant property of GluR-B was traced to the arginine (R) residue at position 586 of the mature subunit, which lies within the pore-forming segment M2 (*4*). This arginine is not gene encoded (*5*) but is posttranscriptionally introduced into GluR-B pre-mRNA (*5*, *6*) by site-selective adenosine deamination, which leads to the change of a CAA glutamine (Q) codon to a CIG codon for arginine in >99% of mRNA molecules (*5*–*7*). Termed Q/R site editing, this nuclear process depends on a double-stranded RNA structure (*6*) formed in the pre-mRNA by the editing site in exon 11 and the editing site complementary sequence (ECS) in intron 11 (*8*). To investigate in an animal model the relevance of this process for central nervous system (CNS) physiology, we targeted intron 11 of the *GluR-B* gene in mouse embryonic stem (ES) cells (*9*) for replacement of the ECS element (*10*) by *loxP* (*11*, *12*) (Fig. 1), and then injected correctly engineered cells into C57BL/6 blastocysts. One of several resultant chimeric animals showed vertical transmission of the *GluR-B*$^{\Delta ECS}$ allele in a Mendelian fashion (*10*), indicating that the allele did not adversely affect embryonic development.

In brains of *GluR-B*$^{+/\Delta ECS}$ mice, the *GluR-B*$^{\Delta ECS}$ allele was expressed with its transcripts remaining unedited at the Q/R site, demonstrated by analysis of allele-specific reverse transcription–polymerase chain reaction (RT-PCR) products (*13*) of partially spliced GluR-B pre-mRNA (Fig. 2A). The sequence and hybridization analysis of RT-PCR products revealed that pre-mRNA derived from the wild-type allele was edited to the expected extent of 83% (*6*, *13*), whereas pre-mRNA from the *GluR-B*$^{\Delta ECS}$ allele, in which the ECS element was replaced by *loxP*, was not edited at the Q/R site. These data showed that the ECS element is indispensable for Q/R site editing in vivo, as previously established for in vitro editing (*6*, *7*). The RT-PCR analysis further indicated that *GluR-B*$^{\Delta ECS}$ pre-mRNA sequences were amplified more efficiently than *GluR-B*$^{+}$ pre-mRNA (Fig. 2A). Quantification with primers that amplify DNA fragments of identical size for both allelic pre-mRNAs (*13*) revealed that premature transcripts of the *GluR-B*$^{\Delta ECS}$ allele are enriched approximately fivefold in the nucleus as compared with premature transcripts of the *GluR-B*$^{+}$ allele. The nuclear accumulation of *GluR-B*$^{\Delta ECS}$ pre-mRNA was attributable to a reduced splicing efficiency of the sequence-modified intron 11, because ribonuclease (RNase) protection with a suitable intron probe (*14*) revealed increased amounts of the *loxP*-containing intron 11 relative to the unmodified intron (Fig. 2B). Consequently, the amounts of cytoplasmic mRNA corresponding to the two alleles were imbalanced, with mature cytoplasmic transcripts unedited at the Q/R site constituting only 25 ± 3% (mean ± SEM, $n = 8$), rather than the theoretically expected 50%, of the GluR-B mRNA population. This situation reflects an overall decrease in GluR-B mRNA abundance, and, indeed, a reduction of ~30% in the amount of GluR-B mRNA was demonstrated by densitometric analysis of Northern

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(RNA) blots (*15*) (Fig. 2C). Other than the imbalance in the mRNAs corresponding to the two alleles, *GluR-B* gene expression appeared normal by Northern analysis, RT-PCR, and in situ hybridization (Fig. 2D). The mutant mRNA was characterized (*13*) by the same flip-flop splice ratios (*16*) and extent of R/G site editing (*17*) as were wild-type transcripts, and the distribution of GluR-B mRNA in the brain was as expected (*18*). In summary, the abundance of GluR-B mRNA in *GluR-B*$^{+/\Delta ECS}$ mice is 70% of that in their wild-type littermates, and one-quarter of these transcripts are unedited at the Q/R site.

In *GluR-B*$^{+/\Delta ECS}$ mice, the functional hemizygosity with regard to Q/R site editing would be expected to result in a shortage of the edited GluR-B subunit for assembly of heteromeric AMPA receptors and, hence, an increase in the glutamate-activated Ca^{2+} permeability of and Ca^{2+} influx through these channels (*19*, *20*). This prediction was confirmed by measurement of responses to fast application of glutamate in nucleated patches isolated from the soma of different neuronal cell types in various brain regions (*21*). For example, in hippocampal pyramidal neurons of the CA1 subfield, the shift of the current reversal potential in a solution containing a high (30 mM) Ca^{2+} concentration to less negative potentials demonstrated that the Ca^{2+} permeability of AMPA receptors in *GluR-B*$^{+/\Delta ECS}$ heterozygotes was 7.3 times that in wild-type homozygotes (Fig. 3). In two other types of principal neurons, cerebellar Purkinje cells and neocortical pyramidal cells, the Ca^{2+} permeability of AMPA receptors in *GluR-B*$^{+/\Delta ECS}$ mice was also increased by a factor of 5.2 to 7.3 (Fig. 3B). Inhibitory basket cells of the dentate gyrus (DG) in *GluR-B*$^{+/+}$ mice showed an average Ca^{2+}/Cs^{+} permeability ratio of 1.2, which is somewhat smaller than that previously estimated from outside-out patch recordings (*22*). This difference is probably attributable to the presence of two classes of basket cells, one of which expresses AMPA receptors with a low Ca^{2+} permeability that give rise to only small ensemble currents (Fig. 3B). These currents could be detected in nucleated patches but not in smaller outside-out patches. Basket cells with lower and higher AMPA receptor–mediated Ca^{2+} permeability occurred also in *GluR-B*$^{+/\Delta ECS}$ mice, in which both groups of cells showed increased Ca^{2+} permeabilities. The difference between the two groups was, however, less pronounced than in *GluR-B*$^{+/+}$ animals (Fig. 3B). Thus, if GluR-B expression is low, as in one class of DG basket cells, the Q/R site–edited subunit would contribute relatively little to the Ca^{2+} permeability of AMPA receptors, and, on average, the increased permeability in the heterozygotes was therefore not as pronounced.

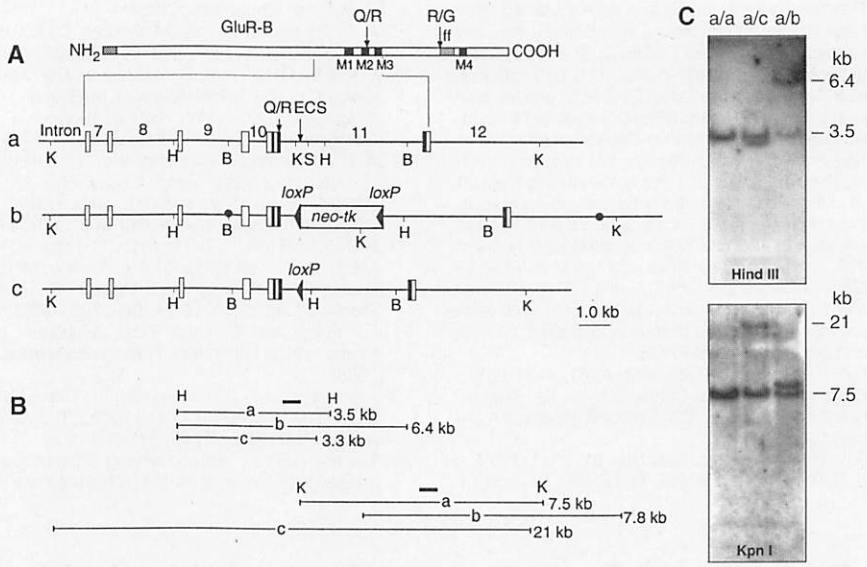

Fig. 1. Generation of *GluR-B*$^{+/\Delta ECS}$ mice. (**A**) Schematic representation of the GluR-B subunit and of gene segments (*8*) of the wild-type *GluR-B*$^{+}$ allele (a), the targeted *GluR-B*^{neo} allele (b), and the *GluR-B*$^{\Delta ECS}$ allele after Cre recombination (c) (*10*). For the protein, Q/R and R/G editing sites (*5*, *6*, *17*) are indicated by arrows; black boxes represent putative membrane segments M1 to M4 (*2*); the hatched box shows the position of the alternatively spliced flip-flop exons (ff) (*16*); and the gray box represents the signal peptide. For the gene segments, open boxes represent exonic sequences (*8*). The *loxP* sites are shown by triangles and the *neo-tk* cassette by an open box. The solid circles in segment b delineate the 5′ and 3′ ends of the targeting construct. Relevant restriction enzyme recognition sites are indicated: K, Kpn I; B, BsrG I; S, Sca I; H, Hind III. (**B** and **C**) Genomic Hind III (H) and Kpn I (K) restriction fragments (B) used in Southern blot analysis (C) to distinguish the *GluR-B* alleles a, b, and c in targeted R1 ES cell clones. Southern probes and their positions (*10*) are indicated by black bars in (B).

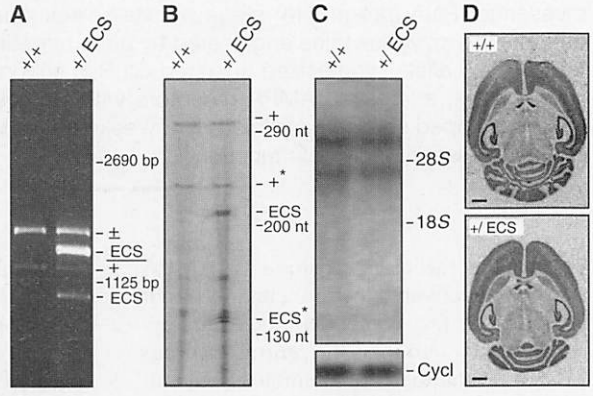

Fig. 2. GluR-B transcript analysis. RNA from the brains of *GluR-B*$^{+/+}$ and *GluR-B*$^{+/\Delta ECS}$ mice was analyzed by various techniques. (**A**) RT-PCR analysis with exon-10 and intron-11 primers (*13*). Amplified fragments containing intron 10 and derived from pre-mRNA or gene sequences are indicated according to allele type by underlined symbols. Amplicons lacking intron 10 and derived from pre-mRNA are denoted by allele symbols that are not underlined, and were cloned for analysis of pre-mRNA. (**B**) RNase protection analysis (*14*). Probe segments protected by the intron-11 sequence in the pre-mRNAs derived from *GluR-B*$^{+}$ and *GluR-B*$^{\Delta ECS}$ alleles are indicated by allele symbols (+, ΔECS). An asterisk indicates probe segments identified as degradation products resulting from overdigestion. Self-protected probe fragments with lower signal intensity are not indicated. (**C**) Northern blot analysis (*15*) with the RNA load controlled for by reprobing the membrane with a probe for cyclophilin mRNA (Cycl). The positions of 28*S* and 18*S* ribosomal RNAs are indicated. (**D**) In situ hybridization of GluR-B mRNA from brains of *GluR-B*$^{+/+}$ (upper panel) and *GluR-B*$^{+/\Delta ECS}$ (lower panel) mice (*18*). Scale bar, 1 mm.

Molecular differences between the *GluR-B*$^{+/+}$ and *GluR-B*$^{+/\Delta ECS}$ animals were revealed in the expression of the Ca^{2+}-responsive immediate-early genes c-*Fos*, c-*Jun*, and *Krox24* (*23*), which likely result from the increased glutamate-activated Ca^{2+} entry into the neurons of heterozygous animals. As observed by in situ hybridization, the abundance of c-*Jun* and *Krox24* (*24*) mRNAs was consistently increased in several brain areas of *GluR-B*$^{+/\Delta ECS}$ mice (Fig. 4A). In particular, c-*Jun* expression was increased in CA1 pyramidal neurons, whereas *Krox24* expression was increased in CA3 pyramidal neurons. Changes in c-*Fos* expression differed among individual heterozygotes, perhaps reflecting the occurrence of spontaneous seizures (see below). In contrast, the abundance of transcripts for AMPA and *N*-methyl-D-aspartate receptor

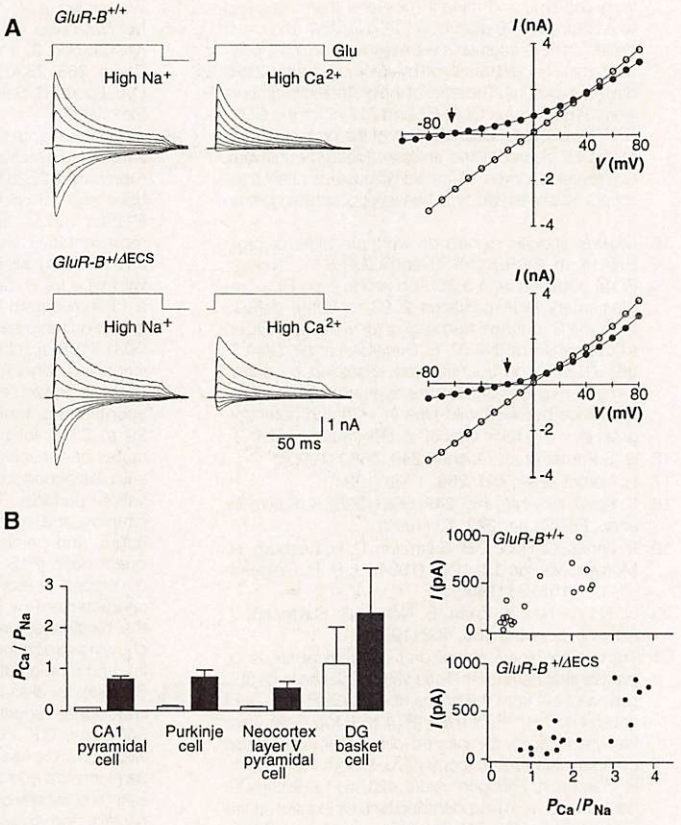

Fig. 3. Relative Ca^{2+} permeability of AMPA receptors in neurons of *GluR-B*$^{+/+}$ and *GluR-B*$^{+/\Delta ECS}$ mice. (**A**) Left panels: AMPA receptor–mediated currents in hippocampal CA1 pyramidal cells evoked by 100-ms pulses of 1 mM glutamate in high-Na^+ and high-Ca^{2+} extracellular solutions, as indicated. The membrane potential was varied between −80 and 80 mV in 20-mV steps. Right panels: Corresponding peak current-voltage (*I-V*) relations for high-Na^+ (○) and high-Ca^{2+} (●) solutions. Arrows indicate the reversal potential in high-Ca^{2+} solution. The *I-V* relations were fitted by third- to sixth-order polynomials, from which the interpolated reversal potentials were calculated. (**B**) Left panel: Ca^{2+} permeability of AMPA receptors in four classes of neurons in *GluR-B*$^{+/+}$ and *GluR*-B$^{+/\Delta ECS}$ mice. Relative Ca^{2+} permeability, expressed as the ratio of Ca^{2+} permeability to Na^+ permeability (P_{Ca}/P_{Na}), is plotted for *GluR-B*$^{+/+}$ (open columns) and *GluR-B*$^{+/\Delta ECS}$ (filled columns) mice. Values are means ± SD of 27 experiments with eight *GluR-B*$^{+/+}$ mice and 46 experiments with seven *GluR-B*$^{+/\Delta ECS}$ mice. Right panels: Plots of relative Ca^{2+} permeability against peak current size at −40 mV in DG basket cells of *GluR-B*$^{+/+}$ and *GluR-B*$^{+/\Delta ECS}$ mice as indicated.

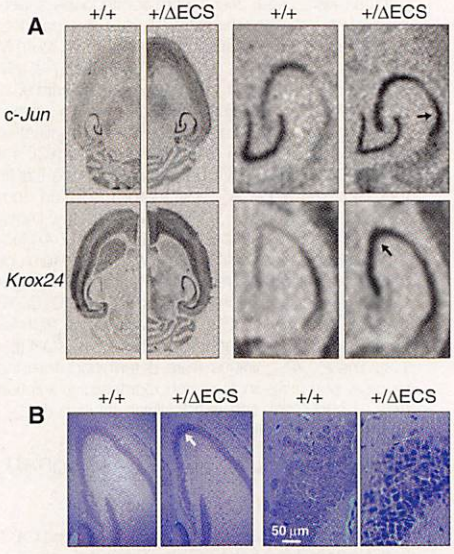

Fig. 4. Histological analysis of brains from *GluR-B*$^{+/+}$ and *GluR-B*$^{+/\Delta ECS}$ mice. (**A**) Autoradiograms of horizontal brain sections (P15) hybridized with ^{35}S-labeled oligonucleotides specific for c-*Jun* and *Krox24* mRNAs (*24*). Half-brain images from wild-type (+/+) and heterozygous (+/ΔECS) mice were apposed to permit better evaluation of expression differences. The hippocampal regions are shown enlarged on the right. Arrows point to the CA3 field for *Krox24* and to the CA1 field for c-*Jun*. (**B**) Hippocampal sections from mouse brains (15 μm), fixed by immersion in 4% paraformaldehyde and embedded in paraffin, were stained with hematoxylin-eosin. The close-ups on the right show the CA3 layer and reveal an increased abundance of eosinophilic cells in the *GluR-B*$^{+/\Delta ECS}$ brain. Arrow indicates the CA3 field.

subunits appeared unaltered (*2*, *16*, *25*).

During the first two postnatal weeks, the heterozygous animals appeared healthy except for an incipient hypotrophy. Beginning at postnatal day 13 (P13), all carriers of the *GluR-B*$^{\Delta ECS}$ allele rapidly developed a severely compromised phenotype, resulting from a neurological syndrome of which the most recognizable manifestations were spontaneous and recurrent seizures, as well as progressively agitated states with excessive jumping and running fits (*26*). All heterozygotes died by P20. Postmortem analysis of the brains of three animals that underwent prolonged seizure episodes revealed selective neuronal degeneration in the lateral region of the hippocampal CA3 field, with ~50% of the neurons showing shrunken nuclei and acidophilic cytoplasmic staining (*27*) (Fig. 4B). Neuronal degeneration was acute in the absence of glial reactions, as assessed by histology and by immunocytochemistry for glial acidic fibrillary protein. Detailed analyses revealed no other apparent abnormalities in the central or peripheral nervous system, and skeletal muscles and visceral organs showed normal histology (*27*). Thus, aberrant excitatory signaling, rather than morphological changes, may underlie the compromised phenotype.

The dominant lethal effect of the *GluR-B*$^{\Delta ECS}$ allele with a penetrance of 100% demonstrates that efficient Q/R site editing of GluR-B pre-mRNA (*5*, *16*) and the resulting low Ca^{2+} permeability of AMPA receptors in excitatory principal neurons (*19*) are pivotal for CNS physiology. Our data suggest that the onset and severity of the epileptic phenotype engendered by a reduction of Q/R site editing depend on the ratio of edited to unedited GluR-B subunits (*28*). It is possible that epileptic mouse models related to altered AMPA receptor properties could be established by regulating this ratio. Indeed, the selective destruction of hippocampal neurons in *GluR-B*$^{+/\Delta ECS}$ mice is reminiscent of kainate-induced hippocampal lesions, which are relevant to human temporal lobe epilepsy (*29*). It remains to be determined whether any of the human familial epilepsies (*30*) derive from related molecular defects.

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10. For construction of the targeting vector pGRB-neotk-1, a BsrG I–Sal I mouse 129/sv genomic fragment containing exons 9 to 12 of the *GluR-B* locus (*8*) was subcloned into pBluescript SK II(−) (Stratagene). The 358–base pair (bp) Kpn I–Sca I genomic fragment of intron 11, which contains the ECS site, was deleted and replaced by a *loxP-neo*^{r}*-tk-loxP* cassette from vector pneotk-1 (R. Sprengel, unpublished data). R1 ES cells (*9*) were electroporated (Bio-Rad Gene Pulser set at 240 V and 500 μF) with 40 μg of pGRB-neotk-1 that had been linearized at the unique Sal I site in the polylinker. Cell clones resistant to G418 (300 μg/ml) selection were isolated after 7 to 10 days. Four targeted clones were identified by nested PCR analysis with rsp27 (5′-AGGACGCGCGGCAAACGAGGGCACCCG-3′) and rsp28 (5′-GCAGGCAAGAGCCGAGGCGAGGCCAAG-3′) as sense primers in intron-9 sequences

outside of the targeting vector and rspneo6 (5′-GCAATCCATCTTGTTCAATGGC-3′) and rsplox5 (5′-CACTGCTCGACCTGCAGCCAAG-3′) as antisense primers of the *neo* cassette. Homologous recombination was confirmed by Southern (DNA) blot analysis (Fig. 1C). To delete the selection marker cassette, 10^7 *GluR-B*$^{+/neo}$ ES cells were electroporated with 30 μg of Cre-encoding plasmid, pMC-Cre (*12*). After 5 to 7 days of ganciclovir (2 μM) selection, the resistant clones were picked and analyzed by PCR with a primer set [sense primer MH53 (5′-GTTGATCATGTGTTTCCCTG-3′) located in intron 11 upstream of the Kpn I site (*6*), and antisense primer rsp36 (5′-CAATAGCAATTGGTGATTTGTGAC-3′) located in intron 11, 3′ of the Sca I site] that amplified a 494-bp fragment of the *GluR-B*$^{+}$ and a 250-bp fragment of the *GluR-B*$^{\Delta ECS}$ allele. Genotypes of *GluR-B*$^{+/\Delta ECS}$ clones were confirmed on Southern blots (Fig. 1C) of Hind III–digested DNA probed with a 400-bp Bgl II–Kpn I fragment derived from exon-11 and intron-11 sequences, or of Kpn I–digested DNA probed with an 800-bp Bgl II–Hpa I fragment encompassing the 3′ portion of intron 11 and exon 12 (*8*). The absence of *neo* [E. Beck *et al.*, *Gene* **19**, 327 (1982)] and *cre* sequences (*11*) in the genome of the clones was confirmed by PCR. Three independent targeted clones that contained the correct sequence replacement were injected into blastocysts of C57BL/6 mice. Of several male chimeras, only one, derived from clone GRB1/50/Cre/13, transmitted the mutated *GluR-B* allele to offspring, whose genotype was determined from tail DNA by PCR analysis with primers MH53 and rsp36 and by Southern hybridization. Of 89 agouti offspring, 43 were *GluR-B*$^{+/\Delta ECS}$.

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13. RT-PCR amplification of GluR-B pre-mRNA sequences from brain tissue [three *GluR-B*$^{+/+}$ animals and six *GluR-B*$^{+/\Delta ECS}$ animals, all P15 to P18] was performed with sense primer rspex10a (5′-GCGGATCCGGAATGAGCGTTACGAGGGCTAC-3′, exon 10) and antisense primer rsp36b (5′-CCAATGCATTTGTGACAAATACTGATAATTAAG-3′, intron 11) or, for quantitative studies, MH36 (5′-TCACCAGGGAAACACATGATC-3′, intron 11). For mRNA analysis, amplification primers B52 (*17*) and 3′lamlo [B. Lambolez, E. Audinat, B. Bochet, F. Crepel, J. Rossier, *Neuron* **9**, 247 (1992)], which are derived from exons 11 and 14-15 (*8*), respectively, were used. For analysis of posttranscriptional modifications, the amplicons (Fig. 2A) were directionally cloned into M13mp19 replicative form DNA and recombinant plaques were analyzed by differential oligonucleotide hybridization (*5, 6, 16, 17*) for Q/R site editing in partially spliced pre-mRNA and mRNA, and for ratios of flip-flop splice forms and of R/G site editing in mRNA. Pre-mRNA was analyzed either from RT-PCR products with primers rspex10a and rsp36b, which differ in size for the *GluR-B*$^{+}$ and *GluR-B*$^{\Delta ECS}$ transcripts and were cloned individually (allele-specific analysis; 17% Q form in *GluR-B*$^{+}$ pre-mRNA, 100% Q form in *GluR-B*$^{\Delta ECS}$ pre-mRNA), or from RT-PCR products with primers rspex10a and MH36, which are identical in size for the two alleles [17% Q form in *GluR-B*$^{+/+}$ mice; 88 ± 3% (mean ± SD, n = 3) Q form in *GluR-B*$^{+/\Delta ECS}$ mice]. The mRNA analysis (Q forms were assigned to mRNA from the *GluR-B*$^{\Delta ECS}$ allele and R forms to the *GluR-B*$^{+}$ allele) revealed 25 ± 3% (mean ± SEM, n = 8) Q form in *GluR-B*$^{+/\Delta ECS}$ mice and <1% Q form in *GluR-B*$^{+/+}$ mice; 50 ± 1% (mean ± SD, n = 3) flip in both *GluR-B*$^{\Delta ECS}$ mRNA and *GluR-B*$^{+}$ mRNA; 71 ± 2% flip R form and 68 ± 5% flop R form in *GluR-B*$^{\Delta ECS}$ mRNA; and 88 ± 2% flip G form and 76 ± 6% flop G form in *GluR-B*$^{+}$ mRNA.
14. For RNase protection analysis [J. M. Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology* (Wiley Interscience, New York, 1994)], a 315-bp Eco RI–Hinc II fragment from intron 11 of the murine *GluR-B* gene (*8*) was cloned in pBluescript II SK(−). The resulting plasmid was linearized at the Xba I site in the polylinker, and antisense RNA was generated by in vitro transcription with T7 polymerase in the presence of [α-^{33}P]uridine 5′-triphosphate. The ^{33}P-labeled riboprobe [369 nucleotides (nt)] was hybridized to 20 μg of total brain RNA from wild-type and mutant mice and then incubated with RNases A (20 μg/ml) and T1 (1 μg/ml). The major protected RNA fragments were resolved on a 6% polyacrylamide gel and visualized by autoradiography (2 to 6 days of exposure). The sizes of the protected fragments were 316 nt for the *GluR-B*$^{+}$ and 217 nt for the *GluR-B*$^{\Delta ECS}$ transcripts. Quantification of the protected fragments by densitometric analysis (MacBAS software, Fuji) revealed a three- to fivefold increase in mutant transcripts relative to wild-type transcripts containing intron 11.
15. *GluR-B*–specific signals on Northern blots of brain RNA from *GluR-B*$^{+/\Delta ECS}$ and *GluR-B*$^{+/+}$ animals (P15) obtained with a 350-bp probe (Eco RI, complementary DNA positions 2583 to 2949) derived from the 3′ untranslated region (*8*) were normalized to cyclophilin mRNA [P. E. Danielson *et al.*, *DNA* **7**, 261 (1988)] and quantitatively assessed by densitometry (Fuji Bas 3000). The average relative signal difference between wild-type (n = 2) and heterozygous (n = 10) mice was 30 ± 9% (mean ± SD).
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21. Transverse hippocampal and neocortical slices or parasagittal cerebellar slices with a thickness of 300 μm were cut from the brains of eight *GluR-B*$^{+/+}$ and seven *GluR-B*$^{+/\Delta ECS}$ mice (P14 to P19). Cells were identified visually by infrared differential interference contrast video microscopy [G. J. Stuart, H.-U. Dodt, B. Sakmann, *Pflügers Arch.* **423**, 511 (1993)]. DG basket cells were also identified by their location at the hilar border of the DG, the triangular shape of their soma, and their discharge pattern (*19*). To increase the size of currents mediated by AMPA receptors, we performed electrophysiological experiments with nucleated patches [W. Sather, S. Dieu- donné, J. F. MacDonald, P. Ascher, *J. Physiol. (London)* **450**, 643 (1992)] that had diameters of 7 to 12 μm. In some experiments with cerebellar Purkinje cells, outside-out patches were used. Slices were continuously superfused with physiological extracellular saline solution, containing 125 mM NaCl, 25 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, that was bubbled with 95% O_2 and 5% CO_2. A Hepes-buffered high-Na^+ extracellular solution, used for perfusing the application pipette, contained 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes-NaOH (pH 7.2). The high-Ca^{2+} extracellular solution contained 30 mM $CaCl_2$, 105 mM *N*-methyl-D-glucamine, and 5 mM Hepes-HCl (pH 7.2). D-2-Amino-5-phosphonovaleric acid (25 μM) was added to block *N*-methyl-D-aspartate receptor channels. The high-Cs^+ intracellular solution contained 140 mM CsCl, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM adenosine triphosphate (disodium salt), and 10 mM Hepes-CsOH (pH 7.3). The P_{Ca}/P_{Na} values were determined from the reversal potentials in Na^+-rich extracellular solution (V_{revNa}) and Ca^{2+}-rich extracellular solution (V_{revCa}) according to the following equation:

$$P_{Ca}/P_{Na} = 0.25\, a_{Na}/a_{Ca}(\exp[(2V_{revCa} - V_{revNa})F/RT] + \exp[(V_{revCa} - V_{revNa})F/RT])$$

where a_{Na} and a_{Ca} are the activities of Na^+ and Ca^{2+} in the extracellular solutions, respectively, and R, T, and F have their conventional meaning [C. A. Lewis, *J. Physiol. (London)* **286**, 417 (1979)]. Activity coefficients were estimated by interpolation of tabulated values (0.75 for NaCl, 0.55 for $CaCl_2$). Both V_{revCa} and V_{revNa} values were corrected for liquid junction potentials of 9.8 and 4.5 mV, respectively.
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26. All heterozygotes developed overt behavioral seizures [R. J. Racine, *Electroencephalogr. Clin. Neurophysiol.* **32**, 281 (1972)]. A total of 14 heterozygous and 10 wild-type mice were observed from P12 for various time periods (8 to 12 hours), and representative video recordings were made. At P13 to P14, all heterozygotes, but none of the wild-type mice, developed recurrent seizures with a "behaviogram" similar to that of kainic acid–induced limbic seizures [J. V. Nadler, *Life Sci.* **29**, 2031 (1981)], characterized by rearing on hindlimbs and concomitant forelimb tremor. In addition to these limbic seizure episodes, animals showed spontaneous tonic-clonic seizures (persisting for 20 to 60 s) followed by drowsiness and altered states of consciousness. On average, generalized seizures occurred two to three times during observation periods. These seizures alternated with jumping and running fits over periods of 2 to 3 hours, and persisted for ~2 days. In the subsequent days (P16 to P20), the heterozygous mice developed a severely compromised phenotype characterized by growth retardation, weakness of the hindlimbs, progressively agitated states with chewing-grooming automatisms, excessive jumping and running fits, and, finally, stupor and death.
27. For histological analyses [B. D. Distrey and J. H. Rack, *Histological Laboratory Methods* (E. & S. Publishers, Edinburgh, U.K., 1970)], two heterozygous and two wild-type mice were perfused transcardially with 4% paraformaldehyde at ages P15, P17, P18, and P19. Brain and visceral organs were dissected, embedded in paraffin, and analyzed as 1-μm sections. Apart from hypotrophy of all organ systems, no gross abnormalities were evident in the central or peripheral nervous system, skeletal muscles, or visceral organs of heterozygotes. In the CNS, all major neuronal populations were present and did not show histological abnormalities, as judged by light microscopic examination of serial sections (1-μm coronal sections every 100 μm) treated with Nissl's stain or hematoxylin-eosin. The cortex of the telencephalon and cerebellum exhibited regular layering. Myelination was also equal in mutant and wild-type animals, as assessed by histochemistry (Klüver-Barrera staining) and immunocytochemistry for myelin basic protein. Marker molecules for specific neuronal subpopulations, including tyrosine hydroxylase, glutamate decarboxylase, parvalbumin, and calbindin [K. D. Beck, L. Powell-Braxton, H. R. Widmer, J. Valverde, F. Hefti, *Neuron* **14**, 717 (1995)], showed the same distribution and were detected in similar numbers of neurons in mutant and wild-type animals. Equal numbers of astrocytes expressing glial fibrillary acidic protein were observed in wild-type and mutant mice, and these cells were particularly abundant in the hippocampus and along white matter tracts.
28. Two chimeric founders derived from the targeted cell line containing the *neo* cassette in intron 11 (Fig. 1A, allele b) gave rise to *GluR-B*$^{+/neo}$ offspring. *GluR-B*$^{+/neo}$ mice had a ratio of Q/R site–edited to –unedited GluR-B mRNA of 10:1 (n = 2), and of 14 heterozygotes only 2 died of seizure-related causes around P30.
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31. We thank B. Sakmann and M. Kiessling for their interest and support; I. Schiller and H. Monyer for critical discussions; M. Higuchi for advice and materials; A. Nagy for the R1 ES cell line; H. Gu for the plasmids pGEM-30, pGH-1, and pMC-Cre; A. Herold for DNA sequencing; U. Amtman for help with in situ hybridization; and R. Pfeffer for animal care. R.B. was the recipient of a doctoral fellowship from the University of Torino, Italy. Supported, in part, by Sonderforschungsbereich grant 317 and funds from the German Chemical Society to P.H.S.

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Pineal Serotonin *N*-Acetyltransferase: Expression Cloning and Molecular Analysis

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Pineal serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, or AA-NAT) generates the large circadian rhythm in melatonin, the hormone that coordinates daily and seasonal physiology in some mammals. Complementary DNA encoding ovine AA-NAT was cloned. The abundance of AA-NAT messenger RNA (mRNA) during the day was high in the ovine pineal gland and somewhat lower in retina. AA-NAT mRNA was found unexpectedly in the pituitary gland and in some brain regions. The night-to-day ratio of ovine pineal AA-NAT mRNA is less than 2. In contrast, the ratio exceeds 150 in rats. AA-NAT represents a family within a large superfamily of acetyltransferases.

Amounts of circulating melatonin increase 10-fold at night in all vertebrates. This rhythm is generated by a variation in the activity of AA-NAT (E.C. 2.3.1.87), the penultimate enzyme in melatonin synthesis (serotonin to *N*-acetylserotonin to melatonin) (*1*). The nocturnal increase in pineal AA-NAT activity also markedly decreases serotonin (*1–3*). The rhythm in melatonin is essential for seasonal reproduction (*4*), modulates the function of the circadian clock in the suprachiasmatic nucleus (SCN), and influences activity and sleep (*5*).

AA-NAT is expressed primarily in the pineal gland and to a variable degree in the retinas of some vertebrates (*6*). The enzyme is rapidly inactivated (half-time, or $t_{1/2} \simeq 3$ min) when animals are exposed to light at night (*1*). Arylalkylamines are strongly preferred as substrates over other amines (*7*). Regulation of AA-NAT activity is controlled by a complex system (*1*) that includes the circadian oscillator in the SCN. At night, the SCN transmits signals to the pineal gland by a neural circuit passing through central and peripheral structures. Light acts on this system through a retinal-to-SCN projection to reset the SCN clock and to gate transmission to the pineal gland. SCN-generated signals stimulate the release of norepinephrine, which acts through α- and β-adrenergic receptors to elevate pineal cyclic adenosine monophosphate and calcium. Studies in rats indicate that these second messengers elevate enzyme activity through a mechanism that requires new gene expression, new protein synthesis, and stabilization (*1*). However, the precise molecular details of AA-NAT regulation are not known, primarily because the complementary DNA (cDNA) encoding AA-NAT was not formerly available.

We cloned AA-NAT by using a cDNA expression library (*8, 9*). Pools of clones were transfected into COS-7 cells that were screened for AA-NAT expression by measuring acetylation of the arylalkylamine 5-methoxytryptamine and of the arylamine phenetidine. This screening identified an AA-NAT clone (clone 87) as indicated by substrate specificity of the encoded protein (Fig. 1) (*10–12*).

The largest amounts of ovine AA-NAT mRNA were found in the pineal gland (Fig. 2). One-quarter as much was found in the retina, consistent with reports of AA-NAT activity in chicken and frog retinas and human retinoblastoma Y79 cells (*6, 13*). We also found that the AA-NAT gene was expressed at low levels in ovine brain and pituitary gland (Fig. 2). This led to the finding of AA-NAT activity in the retina,

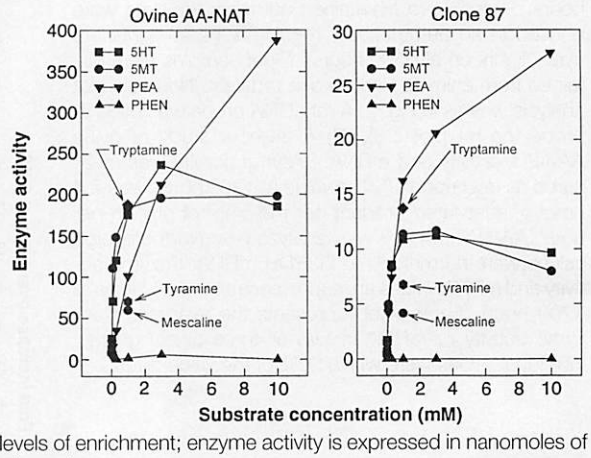

Fig. 1. Substrate specificity of partially purified ovine AA-NAT (*11*) compared with a homogenate of COS-7 cells expressing clone 87. Acetylation was determined in 20-μl assays containing the indicated concentration of amine and 40 μM [^{14}C]acetyl CoA (60 Ci/mol). [^{14}C]Acetylated 5-methoxytryptamine (5MT), tryptamine, phenethylamine (PEA), and phenetidine (PHEN) were extracted into chloroform (*2*). [^{14}C]Acetylated mescaline, serotonin (5HT), and tyramine were resolved by thin-layer chromatography (*10*) with acetylated standards (*27*). Radioactivity was determined by scintillation counting. Different V_{max} values (enzyme activity) reflect different levels of enrichment; enzyme activity is expressed in nanomoles of acetylated product per milligram of protein per hour.

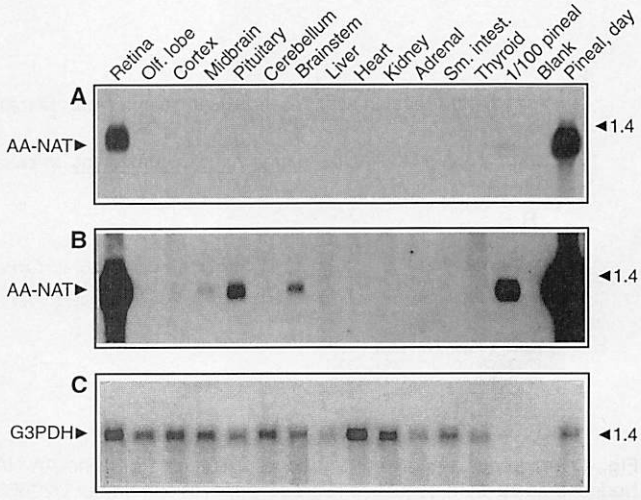

Fig. 2. Extrapineal expression of the gene encoding AA-NAT. Ovine tissue was removed, 1-cm cubes were prepared, and cubes were rapidly frozen. Total RNA was extracted, and Northern (RNA) blot analysis was performed with random-primed cDNA (*16*). Each lane was loaded with 20 μg of total RNA, except the lane marked "1/100 pineal," which received 0.2 μg. Blots were probed with a full-length cDNA insert (960 bp) from clone 87, stripped, and reprobed with G3PDH cDNA to monitor loading and degradation. Similar results were obtained in two additional studies. Olf. lobe, olfactory lobe; Sm. intest., small intestine. Size markers are shown on the right in kilobases. (**A**) A 12-hour autoradiographic image of the blot probed with the insert from clone 87. No other bands of radioactivity were detected. (**B**) A 96-hour autoradiographic image of the same blot. Faint 3- and 5-kb bands were detected inconsistently in pineal glands and retinas (not shown). (**C**) A 12-hour PhosphorImager exposure of the blot probed with G3PDH.

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pituitary, and olfactory lobe (0.5, 0.2, and 0.1 nmol of acetylated product per milligram of protein per hour, respectively) (*14*). The discrepancy between amounts of AA-NAT mRNA and enzyme activity in extrapineal sites may reflect different levels of translation or posttranslational activation. Extrapineal AA-NAT is of special interest because it could influence serotonin concentrations and metabolism.

Ovine pineal AA-NAT mRNA increased less than twofold at night (Fig. 3A), which was much smaller than the nocturnal sevenfold increase in AA-NAT activity (Fig. 3B). In rats, pineal AA-NAT mRNA was very low during the day and increased more than 150-fold at night (Fig. 3A) (*15*, *16*), similar to the increase in enzyme activity (*1*). The rat pineal AA-NAT mRNA rhythm is circadian and is regulated by the same neural pathway that controls enzyme activity (*15*).

These observations indicate that posttranscriptional mechanisms dominate regulation of AA-NAT activity in sheep, whereas in rats both transcriptional and posttranscriptional mechanisms are involved (*1*, *17*, *18*). The involvement of different molecular strategies among species to increase amounts of melatonin at night underscores the biological importance of this signal. These different regulatory mechanisms may explain why in sheep melatonin increases at night immediately after exposure to darkness, whereas in rats there is a delay (*19*). This delay could be linked to the time required to elevate AA-NAT mRNA.

Clone 87 contains a putative 621–base pair (bp) open reading frame (ORF) encoding a 23.1-kD protein (Fig. 4A). The predicted size was confirmed by expression of clone 87 (*20*). Putative regulation sites exist (Fig. 4A): the two cyclic nucleotide–dependent protein kinase phosphorylation sites may be involved in rapid inactivation (*1*, *17*); the two protein kinase C phosphorylation sites may mediate the effects of calcium (*21*), and one or more of the seven cysteines may be involved in activation and inactivation (*18*).

The AA-NAT amino acid sequence was not strongly similar to any functionally characterized protein in the databases; however, it was statistically similar to an uncharacterized, hypothetical yeast protein (YD8554.04c) (*22*). Marginal similarity was also found to *Streptomyces lipmanii* puromycin acetyltransferase (PUAC_STRLP) and a hypothetical protein (ORF5/R46) (Fig. 4B). The similar regions included the portion of AA-NAT that aligned with YD8554.04c, which suggests that these regions may be functionally relevant. This interpretation is supported by the conservation in PUAC_STRLP and AA-NAT of two essential motifs (designated A and B in Fig. 4B) found in several acetyltransferases (*23*). These motifs may function in acetyl coenzyme A (CoA) binding, acetyl group transfer, or both (*24*).

We searched for other AA-NAT–related proteins by using a block that encompasses motif A from the alignments of AA-NAT with the three proteins described above (*25*). Of the 79 related proteins identified in this search, 30 are acetyltransferases and the remainder are uncharacterized, hypothetical proteins. Therefore, it appears that motif A is a reliable identifier of an acetyltransferase superfamily. Subsequent multiple-alignment analysis confirmed the statistical significance of the similarity between AA-NAT and other acetyltransferases and identified additional re-

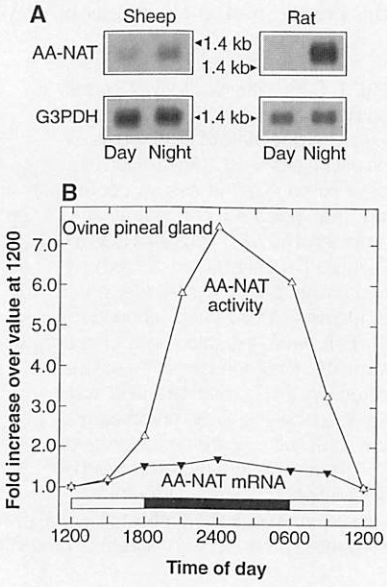

Fig. 3. Day and night studies of pineal AA-NAT mRNA. (**A**) Northern blot analysis. Day pineal glands were removed at ~1200 hours and night pineal glands at ~2400 hours. Sheep were maintained outdoors (*8*). Rats were housed in an automatically regulated 14:10 light-dark cycle (lights on at 0500 hours). Night samples were obtained from animals killed in dim red light. Northern blot analysis was as in Fig. 2. A rat cDNA probe was used to probe the rat blot (*28*). (**B**) A 24-hour study of ovine AA-NAT activity and mRNA. Enzyme activity was measured as described (*2*). The value at 1200 hours was 7.4 nmol of acetylated product per milligram of protein per hour. AA-NAT mRNA was analyzed by Northern blot; values were normalized to G3PDH mRNA. Enzyme activity and mRNA values are expressed relative to values at 1200 hours. Each point represents the average of enzyme activity or mRNA in two or three pineal glands; individual values were within 20% of the value given.

A

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  1 MSTPSVHCLK PSPLHLPSGI PGSPGRQRRH TLPANEFRCL TPEDAAGVFE IEREAFISVS GNCPLNLDEV QHFLTLCPEL SLGWFVEGRL VAFIIGSLWD
                                                *                  +                                    +
                          Motif A                                                 Motif B                          *
101 EERLTQESLA LHRPRGHSAH LHALAVDRSF RQQGKGSVLL WRYLHHVGAQ PAVRRAVLMC EDALVPFYQR FGFHPAGPCA IVVGSLTFTE MHCSLRGHAA LRRNSDR
                          #                                                                    +          #          #
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B

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Consensus                                                                                    Motif A          Motif B
                                                                                             U..UOU.P.UQ..GUO$.UU   U.UY..UGU....
AA-NAT (U29663)      36 FRCLTPEDAAGVFEIEREAF 17 LTLCPELSLGWFV  3 LVAFIIGSLWDEERLTQESLA 1 HRPRGHSAH 0 LHALAVDRSFRQQGKGSVLL 24 VPFYQRFGFHPAG 30
                           :* *  **   ::::* ::*    *  **** * *:    *:: *:*:    * :* **::      : * *   :*:: :   :     :::**    :***:*:**   :
YD8554.04c (S49826)  13 IRPLIIEDLKQILNLESQGF 14 LINCPELCSGLFI 12 LIGHIMGTKIPHEYITIESMG 1 LQVES-SNH 2 IHSVVIKPEYQKKNLATLLL 24 IPFYERVGFKIIA 30
PUAC_STRLP (P13429)  93                                                RMAELSGSRLAAQQQMEGLLA 9 HRPKEPAWF 0 LATVGVSPDHQGKGLGSAVV 23 LPFYERLGFTVTA 19
AAC6_CITDI (P10051)  78                                                                                  LHPLVVRPDYQNKGIGKILL 52 YEFYQKNGYYIVG 22
RIMI_ECOLI (P09453)  65                                                                                  LFNIAVDPDYQRQGLGRALL 26 IALYESLGFNEAT 37
SAT4/CAMCO (U01945)  98                                                                                  IEDIAVCKDPRGQGIGSALI 25 CKFYHNCGFKIGS 23
AAC1_PSEAU (P23181) 107                                                                                  IYDLAVSGEHRRQGIATALI 26 VALYTKLGIREEV 11
YJHQ_ECOLI (P39368)  81                                                                                  LAPLAVIPEYQGMGVGGRLI 20 ATYYPRHGFEPCA 47
ORF5/R46 (507658)    92                                                                                  LHKLAVRRTHAGRGVSSALI 23 RGLYERLGFTHVD 15
ORF/STAAU (S26352)   35                                                                                  LHSIGMLPNYQDKGYGSKLL 26 CHVYEKLGGKNDY 15
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Fig. 4. Amino acid sequence and conserved motifs of ovine AA-NAT. (**A**) Deduced amino acid sequence (GenBank accession number U29663) (*29*). Putative phosphorylation sites: *, cyclic nucleotide–dependent; #, protein kinase C; +, casein kinase II. The two conserved motifs are bold and underlined. (**B**) Alignment of the AA-NAT amino acid sequence with sequences of known and putative acetyltransferases (*25*). The number of amino acid residues from the protein termini and between the aligned blocks are indicated. Identities between AA-NAT and YD8554.04c are indicated by asterisks; similarities, by colons. Consensus residues are conserved in most aligned sequences (bold residues conform to the consensus): U, bulky hydrophobic residue (I, L, V, M, F, Y, W); O, small residue (G, A, S); $, S or T; dot, any residue. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

gions of limited similarity between AA-NAT, YD8554.04c, and PUAC_STRLP (Fig. 4B) (*25*).

Although motifs A and B define a large acetyltransferase superfamily, this superfamily excludes arylamine *N*-acetyltransferase and many other acetyltransferases (*23*, *26*). AA-NAT appears to represent a new family within this superfamily because there are no closely related proteins in the databases. The AA-NAT family may have emerged relatively late in evolution, perhaps in association with the evolution of melatonin as a photochemical signal. The cloning of AA-NAT opens the door to research on the function of extrapineal AA-NAT and should lead to the resolution of many long-standing issues in research on circadian rhythms and photoperiodism.

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8. Timed ovine pineal glands were purchased from the University of Auckland Medical School, Auckland, New Zealand. Animals were maintained outdoors and housed indoors (12 hours light:12 hours dark) for 1 day. Other tissues and pineal glands were obtained from the University of Michigan and were from adult sheep killed at ~1200 hours.
9. Size-selected (>1 kb) oligo(dT)-primed cDNA, prepared from polyadenylated RNA from the pineal glands of night-killed sheep, was used to construct a unidirectional cDNA library (1.7×10^6 independent recombinants) in the eukaryotic expression vector Zap Express (Stratagene, La Jolla, CA). pBK-CMV phagemids were excised from pools of 1000 phage from the unamplified library and transfected into COS-7 cells with Lipofectamine (Gibco/BRL, Gaithersburg, MD). After 48 hours, the cells were harvested and assayed for arylamine *N*-acetyltransferase and AA-NAT activities (*10*). Three positive pools were detected out of the first 50,000 clones; one was purified to a single colony (clone 87).
10. Samples were incubated with amine substrate and 40 μM [^{14}C]acetyl CoA (60 Ci/mol) in 0.1 M sodium phosphate buffer, pH 6.8, for 1 hour. One volume of ethanol containing 1 mM HCl, 1 mM authentic acetylated product, and 0.001% ascorbic acid was added, and the acetylated product was resolved by thin-layer chromatography (PE SIL G/UV sheets, Whatman, Maidstone, England; 30 min in chloroform-methanol-NH_4OH, 80:20:1). The acetylated products were located, and radioactivity was determined (PhosphorImager, Molecular Dynamics, Sunnyvale, CA).
11. Glands were homogenized (1:10 w/v) at 4°C by use of a Polytron (Brinkmann Instruments, Westbury, NY) in 5 mM citrate, pH 5.5, containing 1 mM dithiothreitol (DTT) and 5% glycerol (buffer A); the homogenate was centrifuged (1 hour, 100,000*g*), and the supernatant was applied (1 ml/min, 4°C) to a WAX column (Zorbax Bioseries, Dupont, Wilmington, DE) equilibrated with buffer A. The adsorbed material was eluted with NaCl (0 to 1 M; 55 min, 1 ml/min) and the eluent assayed for AA-NAT activity (*2*). Chromatography enriched AA-NAT activity 10-fold and removed arylamine *N*-acetyltransferase.
12. The apparent K_m (Michaelis constant; acetyl CoA = 40 μM) values for 5-methoxytryptamine with partially purified pineal AA-NAT and with expressed AA-NAT were 90 and 120 μM, respectively; those for 5-hydroxytryptamine were 180 and 140 μM, respectively. Neither enzyme preparation acetylated 1 mM aniline, sulfamethazine, 2-aminofluorene, isoniazid, *p*-aminobenzoic acid, octopamine, dopamine, norepinephrine, epinephrine, amphetamine, histamine, tryptophan, puromycin, glucosamine, ethanolamine, or chloramphenicol.
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14. Tissues (Fig. 2) from two animals were homogenized in 10 volumes of 0.1 M sodium phosphate buffer, pH 6.8, containing 1 mM DTT, 2 μM leupeptin, 0.5 mM *p*-aminoethylbenzenesulfonyl fluoride, and 1 mM sodium fluoride. The homogenates were assayed in duplicate (*2*, *10*); mean values are given. Pineal AA-NAT activity was 22 nmol of acetylated product per milligram of protein per hour.
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22. The comparison was done with the BLASTP program [S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990)]. The probability of AA-NAT and YD8554.04c matching by chance is $\sim 10^{-4}$; there is 31.7% identity and 60.3% similarity in two aligned segments of a total length of 63 amino acid residues.
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25. Database screening with position-dependent weight matrices derived from alignment blocks was performed with the MoST program [R. L. Tatusov, S. F. Altschul, E. V. Koonin, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12091 (1994)]. The search was performed iteratively with the cutoff set as $r = 0.02$. Subsequently, alignments were analyzed with the MACAW program [G. D. Schuler, S. F. Altschul, D. J. Lipman, *Proteins Struct. Funct. Genet.* **9**, 180 (1991)]. The probability of obtaining the observed level of similarity by chance in the 10 aligned sequences was less than 10^{-19} for both motif A and motif B.
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Plecopteran Surface-Skimming and Insect Flight Evolution

The origin of insect flight has been fertile ground for hypotheses (*1*). A recent addition to this debate is the hypothesis of James H. Marden and Melissa G. Kramer that skimming on the water surface in stoneflies represents an intermediate stage in insect flight evolution (*2*). This hypothesis is based on three components: (i) observed skimming behavior in *Taeniopteryx burksi* (Plecoptera: Taeniopterygidae); (ii) experiments showing improved skimming performance with increases in size of wings and power of the flight motor; and (iii) phylogeny suggesting this is ancestral for Plecoptera and all pterygote (winged) insects.

Observed skimming behavior, and experimental results supporting improved skimming performance with size and power increase, are not sufficient reasons to accept skimming as a precursor to flight. As with any evolutionary scheme, a character, morphological (*3*) or behavioral (*4*), must be assigned polarity on the basis of ancestor-descendant relationships to have value for phylogenetic inference.

Marden and Kramer suggest that Plecoptera represents a basal insect order. They state that the extant insect orders Ephemeroptera and Plecoptera are anatomically and phylogenetically closest to pre-flight fossil insects and that stoneflies are generally archaic in their morphology. However, phylogenetic studies supporting this opinion are not presented. Major works on the insect orders refute this hypothesis (*5–8*). Plecoptera represent a potentially basal group in Neoptera, either together with other "orthopteroid" orders (*6, 8*) or with close affinities to Embioptera or Dermaptera (*5–7*). Even if Paleoptera represents an evolutionary grade paraphyletic to Neoptera, there is a clear distinction between Paleopteran and all Neopteran orders based on wing and genitalic morphology (*5, 6*). Phylogenetic and fossil evidence support an origin of flight in a common ancestor of Neoptera + Paleoptera, not in a common ancestor of Plecoptera versus other Pterygota. Falcate wing hairs, cited by Marden and Kramer as homologous in Plecoptera and Ephemeroptera, represent convergence on hydrofuge-hair surfaces analogous to those found in Trichoptera and Diptera (*9*). Ephemeropteran "skimming," referred to by Marden and Kramer, whether subimago behavior when escaping accidental water contact or "skimming" of some adults during mating flight (*10*), cannot be interpreted as homologous to skimming in *T. burksi* on the basis of established methods for determining homologies (*4*).

Marden and Kramer also use phylogenetic relationships within Plecoptera to support the plesiotypic nature of skimming, citing Nelson (*11*), Brodsky (*12*), and Zwick (*12*) for evidence of a basal position for the superfamily Nemouroidea, which contains *Taeniopteryx*, in Plecoptera. Nelson (*11*) places Nemouroidea as sister to the Systellognatha (including Perloidea) and derived relative to Gripopterygoidea and Eustnemioidea. The position of Nemouroidea is clearly not basal in this analysis. Nelson (*11*) discusses the works of Brodsky (*12*) and Zwick (*12*) and considers them "closely congruent" with his own. I reanalyzed Nelson's matrix, eliminating uninformative autapomorphies and recoding nonindependent characters. My consensus tree places Nemouroidea as a relatively derived superfamily (Fig 1A). Thus, Marden and Kramer's hypothesis "that this lineage has never contained more than marginal fliers . . ." must be rejected, or we must accept multiple origins of flight within insects, several times within Plecoptera alone (Fig. 1, A and B). The alternative, a reappropriation of the existing flight apparatus, would require only a single additional step.

Phylogenetic evidence does not support the hypothesis that the flight apparatus in Plecoptera is plesiomorphic. Marden and Kramer's hypothesis is not based on sound phylogenetic analyses and has resulted in a misconstrued evolutionary scenario. Skimming behavior in stoneflies is an evolutionary terminal of its own and not an ancestral state. Surface skimming can be added to the list of feasible scenarios put forward (*1*), but without the support of phylogeny it remains speculative.

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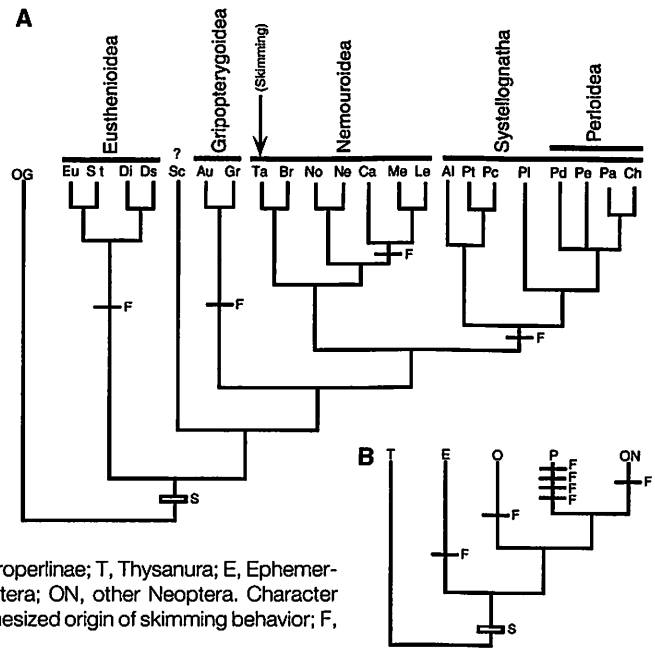

Fig. 1. Cladograms showing distribution of steps required to support the "skimming" hypothesis. (**A**) Consensus cladogram from two trees from recoded matrix. (**B**) Cladogram of derived orders of Insecta. OG, outgroup; Eu, Eustheniinae; St, Stenoperlinae; Di, Diamphipnoa; Ds, Diamphipnopsis; Au, Austroperlidae; Gr, Gripopterygidae; Sc, Scopuridae; Ta, Taeniopteryginae; Br, Brachypterinae; No, Notonemouridae; Ne, Nemouridae, Ca, Capniidae; Me, Megaleuctrinae; Le, Leuctrinae; Pc; Pteronarcella; Pt, Pteronarcys; Al, Allonarcys; Pl, Peltoperlidae; Pd, Perlodidae; Pe, Perlidae; Pa, Paraperlinae; Ch, Chloroperlinae; T, Thysanura; E, Ephemeroptera; O, Odonata; P, Plecoptera; ON, other Neoptera. Character state transformations: S, hypothesized origin of skimming behavior; F, minimum origins of flight.

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19 April 1995; accepted 24 August 1995

Response: Our study of surface skimming locomotion in stoneflies (*1*) has apparently run afoul with systematists because of a poorly worded sentence in our introduction. We stated (*1*, p. 427) that previous studies have not "utilized detailed examinations of . . . the extant insect orders (Ephemeroptera and Plecoptera) that are anatomically and phylogenetically closest to pre-flight fossil insects." The purpose of this sentence was to briefly reflect the current view that these two orders are both near the base of the as yet poorly resolved phylogeny of winged insects (*2*), and that they possess many morphologically primitive traits. The latter notion is not our "opinion," but rather comes from two authoritative 1994 reviews (*3*). We did not mean to imply that Ephemeroptera and Plecoptera are sister taxa, or that either of these orders are ancestral to any other flying insects, but we agree with Will's statement that it is the common ancestor of paleopterous orders (such as Ephemeroptera) and neopterous orders (such as Plecoptera) whose locomotor habits are of interest for resolving the mystery of insect flight evolution.

Because the common ancestor of mayflies and stoneflies first evolved wings, traits that are homologous in these two orders are of particular interest. Thus, our demonstration of morphologically similar water-resistant hairs on the wings of stoneflies and mayflies hints that their common ancestor may have used its wings in a semi-aquatic setting. The fact that other semi-aquatic insects also have these hairs has apparently led Will to the unfounded conclusion that this must reflect evolutionary convergence. We know of no data with which to resolve the issue of homology or convergence for wing hairs.

We described the Nemouridea superfamily (which contains our subject species) as "near the base of the Plecopteran lineage," which Will has misrepresented as a claim of "basal." The distinction is made insignificant by the fact that there is no strongly supported phylogeny for stoneflies. Basal position of the Eusthenioidea depends on questionable behavioral and morphological characters (*4*), which places the rooting of the tree in considerable doubt. Because nearly equally parsimonious cladograms yield fundamentally different phylogenies, Nelson concluded (*5*, p. 472) that "the present character data are not adequate for resolving phylogenetic relationships within Plecoptera." Even if a well-supported Plecopteran phylogeny existed, mapping flight ability onto it would be difficult. For all groups except those that we have recently studied, the literature contains only sparse and imprecise descriptions of flight ability. On the basis of the few descriptions available, the only stoneflies that might come close to being strong fliers are certain species in the Systellognatha and Perloidea, the most derived Plecopteran groups.

The earliest known Plecopteran fossils (*6*) (260 million years old) include the family Taeniopterygidae, whose wings show a less complex pattern of venation (the structural support elements that resist deformation during loading by aerodynamic forces) than modern *Taeniopteryx*; thus it appears that surface-skimming stoneflies are an ancient lineage whose wing evolution may have been progressive rather than digressive during the past quarter-of-a-billion years. Of the two species whose flight capacities we have examined (*1*, *7*), one (*Allocapnia vivipara*) cannot fly except by very crude gliding, whereas certain individuals of the other species (*T. burksi*) can fly by wing-flapping when their muscle temperature is sufficiently warm. Thus, these insects blur the distinction between flying and nonflying and demonstrate that flight is but one extreme on a continuum of aerodynamically powered locomotion, whose evolutionary origin may have been for horizontal movement across water, and whose ancestral state might be retained in lineages that never reached the end of the continuum. If indeed the original radiation of winged insects was among early surface skimmers, true flight would have evolved independently in various lineages, and perhaps even within modern stoneflies. Viewed in this light, the multiple origins of flight shown in figure 1B of the comment by Will is a pattern one would expect. A strict reliance on parsimony to guide our interpretation would blind us to a pattern of gradual transition along the various levels of a quantitative trait.

It remains uncertain whether surface skimming stoneflies represent a loss-of-flight or have retained a plesiomorphic condition. Our purpose in pointing out the latter possibility was to put forth a radically different interpretation from the "monophyletic origins of insect flight" scenario that had become a nearly unquestioned paradigm, and to thereby stimulate further functional and phylogenetic analyses that may resolve the issue.

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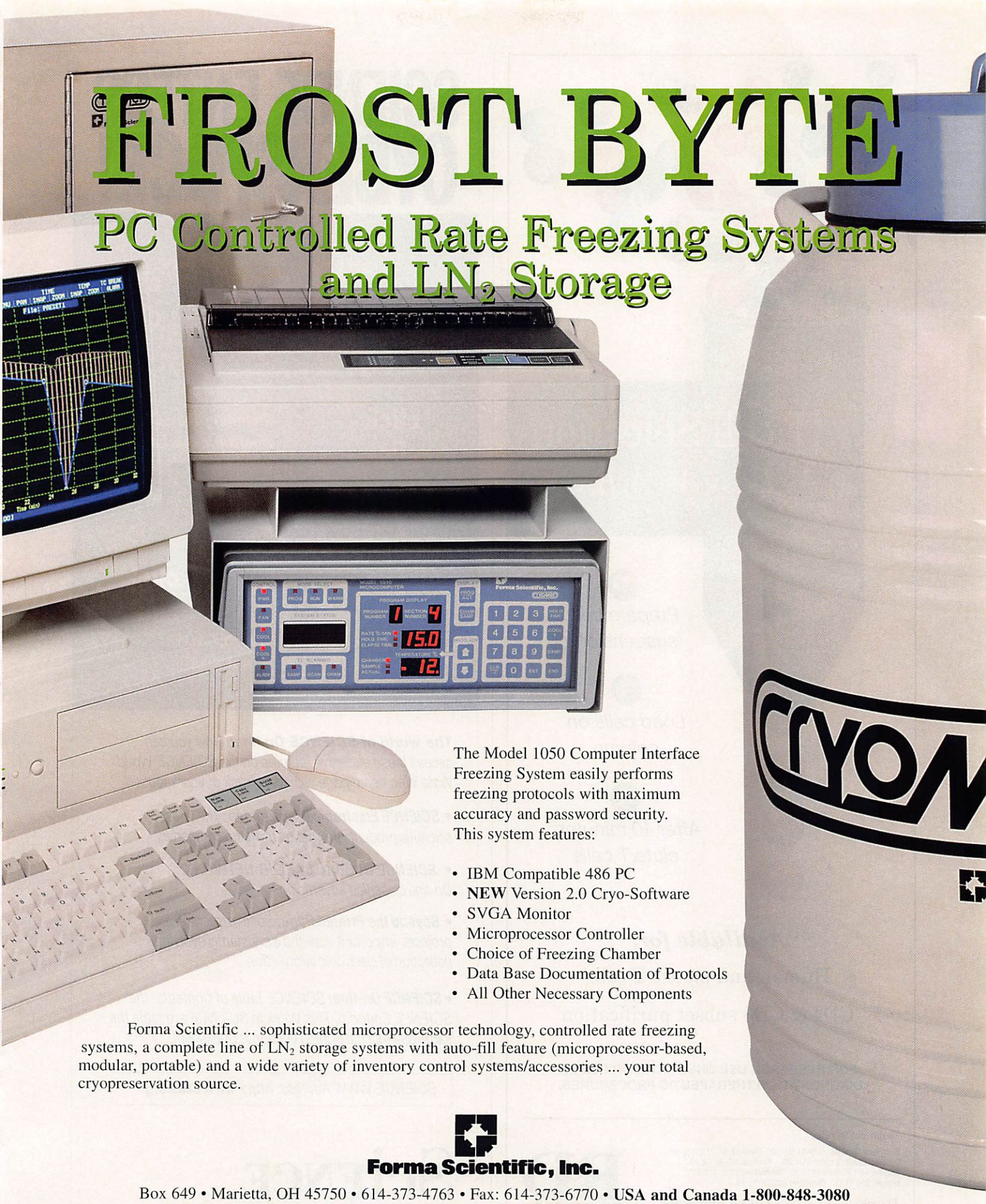

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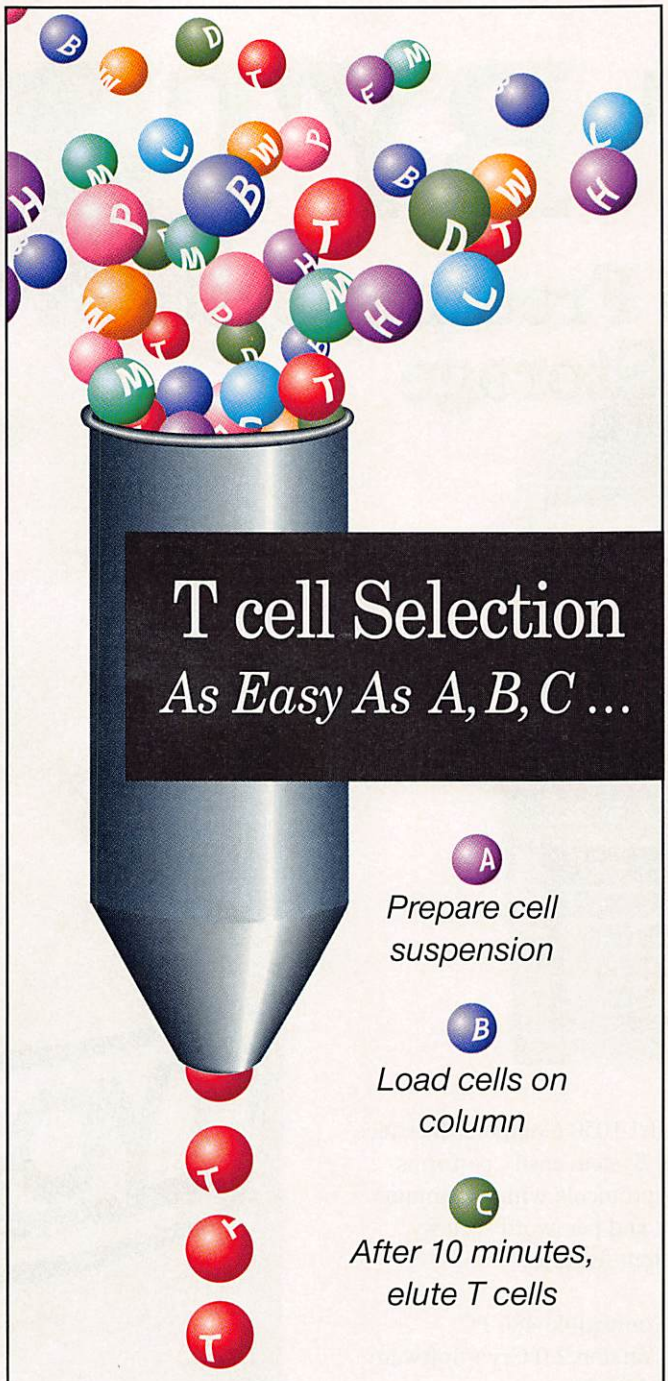

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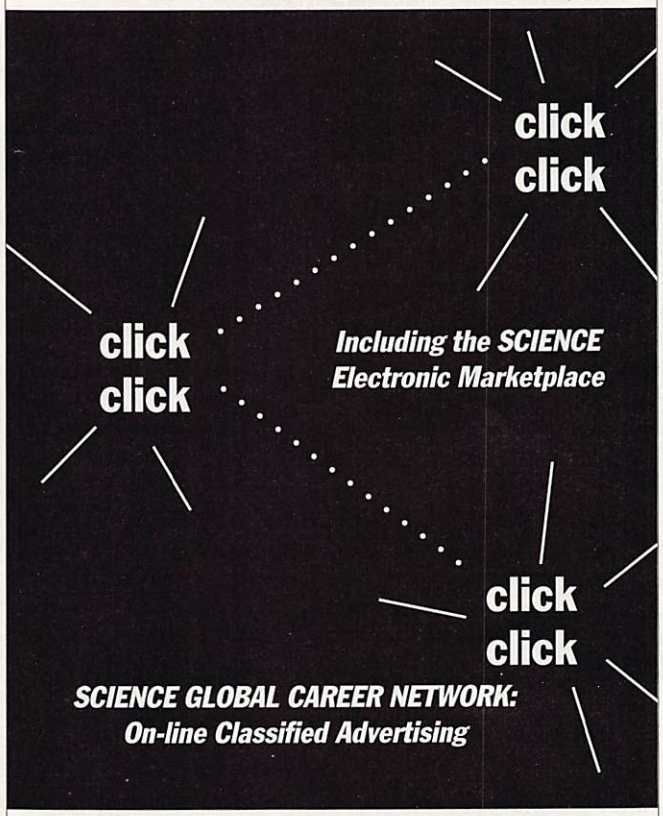

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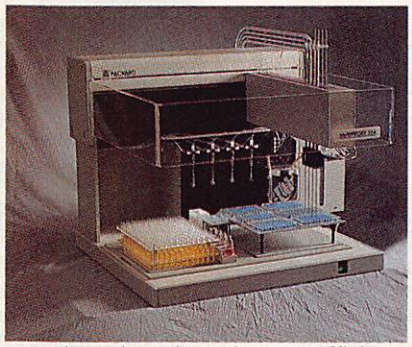

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BOOK REVIEWS

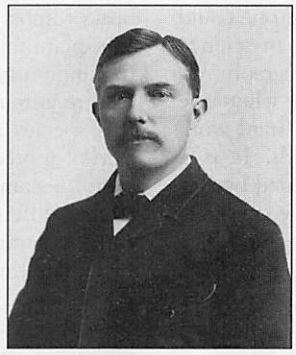
A Committed Observer

The Immortal Fire Within. The Life and Work of Edward Emerson Barnard. WILLIAM SHEEHAN. Cambridge University Press, New York, 1995. xiv, 429 pp., illus. $49.95 or £40.

The life of E. E. Barnard (1857–1923) straddled an era of revolutionary change in astronomy, especially in the application of photography and spectroscopy to the heavens. It was in photography that Barnard did his pioneering work, leaving a legacy of thousands of photographic plates, including the earliest wide-field images of the Milky Way. Given the impoverished circumstances of his early life, Barnard's achievements are remarkable, and with this biography William Sheehan has given us the first detailed account of Barnard's step-by-step journey toward scientific prominence.

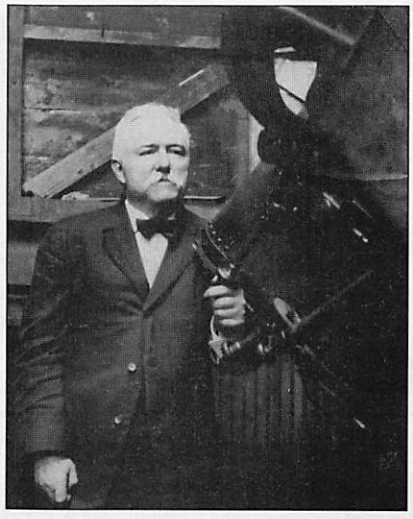
E. E. Barnard pictured with view wagon operated in the 1870s by James W. Braid for Poole's Photographic Gallery, Nashville, Tennessee. Barnard was Braid's assistant in outdoor photography. [From *The Immortal Fire Within*; Special Collections, Vanderbilt University]

Barnard's life falls into three distinct eras: his early life and work in Nashville, Tennessee (1857–1887), his observations at the newly constructed Lick Observatory in California (1887–1895), and his culminating achievements at the Yerkes Observatory in Williams Bay, Wisconsin (1895–1923). In Nashville at the age of nine he went to work for a local photographer, and by 1877 (the year Asaph Hall discovered the moons of Mars with a 26-inch refractor) Barnard had purchased a 5-inch telescope. Also in that year Barnard took the opportunity to ask Simon Newcomb—president-elect of the AAAS and presiding officer at its meeting that year in Nashville—for his advice to an aspiring astronomer. A stern Newcomb counseled Barnard to abandon the telescope until he understood mathematics; luckily Barnard—who had no aptitude for higher mathematics—largely ignored Newcomb's advice, and by 1879 he began systematic observations with his small telescope. By the time he left Nashville eight years later, Barnard had nine comet discoveries to his credit, had brought astronomical acclaim to the fledgling Vanderbilt University (which had an observatory with a 6-inch refractor), and was well known among American astronomers. All of this he accomplished with very little formal training, utilizing only keen eyesight and determination that became legendary.



E. E. Barnard in 1893. [From *The Immortal Fire Within*; Mary Lea Shane Archives, Lick Observatory]

With this background, in 1887 Barnard landed a job at Lick Observatory, where the 36-inch refractor was nearing completion. His junior status left him largely to work on the 12-inch telescope, however, and increasingly he was at odds with Lick director E. S. Holden. Fully half of Sheehan's book is devoted to Barnard's eight years at Lick, and, with Donald Osterbrock's history of Lick Observatory and biography of Barnard's contemporary James E. Keeler, this period of Lick's history is now well understood. Barnard discovered more comets during his years in California, but more important, on 9 September 1892, a few months after he was given regular use of the larger telescope, he discovered a fifth moon of Jupiter (now known as Amalthea), which was to be the last of the visually discovered moons of the solar system. This discovery further added to Barnard's reputation for keen eyesight. So also did his observations of Mars; Barnard's conservative attitude toward the interpretation of observations prevented excesses such as Percival Lowell's claim to have found canals. Ironically, the same caution delayed for many years a correct interpretation of the results of Barnard's photographic work on the Milky Way, begun at Lick using a wide-field 6-inch "Willard lens."



E. E. Barnard with the Bruce Photographic telescope. [From *The Immortal Fire Within*; Yerkes Observatory]

Engaged as he was with visual and photographic techniques in astronomy, Barnard also worked in an era when reflectors would gradually supersede refractors. In 1895, the year he left Lick for Yerkes with its mammoth 40-inch refractor (still the largest in the world), Lick obtained a 36-inch reflector, and by 1900 Keeler demonstrated its superiority for photographing spiral nebulae (which many astronomers still believed to be planetary systems in formation). Barnard, however, remained firmly in the refractor tradition.

During his Yerkes years Barnard photographed every comet visible from Yerkes, producing some 1400 plates. He also discovered the nearby and fast-moving star that now bears his name. But most of all Barnard continued and consolidated his photographic work on the Milky Way, and after long consideration came to the conclusion that many of the "holes" and patterns evident in his photographs were actually dark obscuring nebulae viewed against the bright background of stars. Barnard's *Comet and Milky Way Photographs* (1913) and *Atlas of Selected Regions of the Milky Way* (1927) encapsulate his life work, and the catalog of dark nebulae contained in the latter was not superseded until the 1960s.

Sheehan, an amateur astronomer and psychiatrist, occasionally makes psychological judgments of Barnard that, though in-

teresting, are unsatisfying because unprovable. But overall he has provided a well-written and meticulously researched biography that makes judicious use of voluminous archives. With its numerous photographs (including many of Barnard's own) it is a handsome and well-produced volume, marred only by a series of mismatches, in chapter 20, between footnote numbers and superscripts in the text.

After Barnard's death one of his Nashville friends spoke of "the immortal fire within" that drove Barnard ruthlessly to observe night after night in his effort to understand the universe, sometimes to the detriment of his personal health and safety. Although it is unlikely today that one could reach the top of any profession in science without formal training, this biography recounts an inspiring example of what one individual can accomplish with hard work and dedication, against all odds. It is a lesson that should not go unheeded by students or professionals; even in the era of big science.

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■

All About Mice

Mouse Genetics. Concepts and Applications. LEE M. SILVER. Oxford University Press, New York, 1995. xiv, 362 pp., illus. $49.95 or £40.

Mapping the human genome and identifying genes that contribute to susceptibility and resistance to disease are currently major preoccupations in biomedical science. The foundations for much of this sophisticated field of research were established in the mouse. Almost a hundred years ago, when it was recognized that laboratory mice developed tumors, attempts were made to use transplantable tumors as a means of inducing immunity to cancer. This led ultimately to a requirement for genetic uniformity in this species and the subsequent construction of inbred strains beginning in 1921. It soon became apparent that individual strains differed in their susceptibilities to cancer. Strains with a high incidence of mammary tumors (C3H) or leukemia (C58, AKR) stood in contrast to low–spontaneous-cancer strains (BALB/c, C57BL). The biological problems evolving from the transplantation of tumors in inbred strains opened a brilliant chapter in mammalian genetics with the discovery of the major histocompatibility locus. But quickly the progress in the mouse led to comparable work in humans and the mouse was relegated to a more parochial role in mammalian genetics.

Several important developments rekindled interest in the genetics of the mouse and made genetic findings more relevant to our understanding of human biology. Molecular geneticists discovered that many genes are highly conserved throughout evolution and that the order of genes in segments of chromosomes is conserved as well. Despite exceptions, "over 80% of the autosomes have now been matched up at the subchromosomal level." This linkage homology has made possible the transposition from one genome to another. Yet another series of observations opened additional ways in which to study human genes in mice. Construction of transgenic mice and gene targeting by homologous recombination to "knock out" the function of genes has created new strains of mice for studying mechanisms of gene action. The combination of these two methods can potentially produce mice that function with human genes.

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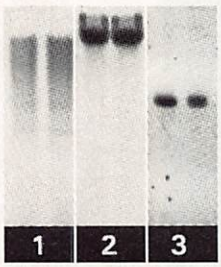

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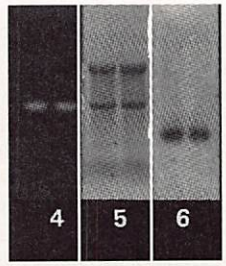

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lecular genetics and the biology of the laboratory mouse. As the house mouse and its genome are sometimes employed or regarded (regrettably) as a genetic "test-tube," there is growing need for the student as well as those who manage colonies of mice to become familiar with the biology of this fascinating creature. Though mundane, "all you ever wanted to know about the sex-life and reproductivity of the house mouse but were afraid to ask" is covered in this book. However, one should not be misled into thinking that the primary mission of the book is to give equal and independent time to husbandry issues along with molecular genetic technology. *Mouse Genetics* is truly a composite in which all elements contribute to a global understanding. It provides exciting reading for the student and continuing education for the more applied worker and above all unifies its topic. There are practical discussions for the student or researcher including such sections as "starting *from scratch* with a new mapping project." In his effort to assist the reader in the "fundamental goal of molecular genetics," Silver describes and evaluates strategies for mutagenesis (chemical, transgene, knock-out) and linkage analysis (using backcross, congenic, and recombinant inbred strains) to dissect how "genotypes *are* translated into phenotypes." Silver's insightful interest in the evolution of genomes surfaces over and over again in the text, making this book an exciting introduction to the comprehensive biology that is involved in the intriguing mysteries of the evolutionary process.

Michael Potter
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■

Interleukins Etc.

Guidebook to Cytokines and Their Receptors. NICOS A. NICOLA, Ed. Oxford University Press, New York, 1995. xvi, 261 pp., illus., + plates. $75 or £45; paper, $39.50 or £22.50. A Sambrook and Tooze publication.

Most books on cytokines are of evanescent value, being rendered obsolete very quickly by the emergence of new ligands, new receptors, new transducers, and new facts. With this thought in mind I looked askance at *Guidebook to Cytokines and Their Receptors*. Surely, I thought, the task of evaluating it would be akin to appraising a computer with a 386 chip. Refreshingly, it was not. True, there are specific areas in which discoveries have eclipsed the material presented. But for the most part the facts and principles set forth are not likely to change. This attests, in part, to the diligence of Nicola and the panel of experts he has enlisted to lay out the information. It also says something about the field as a whole, which seems at last to have reached a point of maturity.

The book begins with an overview of the cytokines, written by Nicola himself, and an overview of their receptors, written by Douglas J. Hilton. The reductionist approach that these authors entertain is most helpful, since only four families of receptors are known to accommodate the dozens of cytokines that exist. It is a bit disappointing to find that the book then presents the cytokines in an order dictated by their numbers, rather than clustering them in groups related to receptor function or structure. What, after all, do the appellations "IL-1, IL-2, . . . IL-*n*" really mean?

The enduring quality of this *Guidebook* owes much to the stylistic consistency and thoroughness of the individual entries. Many, though not all, of the cytokines have

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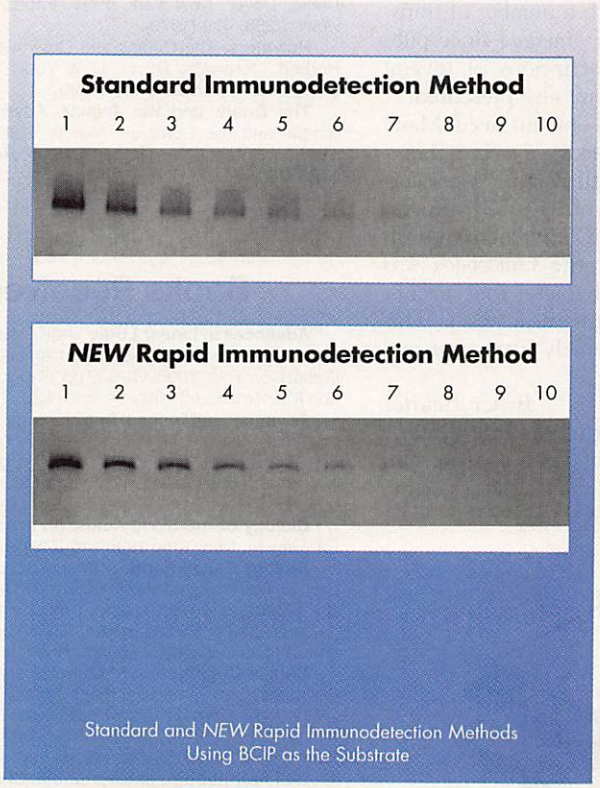

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Vignettes: Confidings of Herpetology

By the late 1980s . . . scientists who work with frogs slowly and cautiously began sharing their tales of frog-finding difficulty with their colleagues. Among herpetologists, particularly those who work in the field more than in laboratories, being able to find amphibians traditionally has been a matter of pride. If a graduate student returned from a summer field trip complaining about not being able to find animals, colleagues and teachers assumed the student was not very competent. So when the professionals began sharing their experiences of not finding frogs, it was almost cathartic, like releasing some horrible family secret.

—*Kathryn Phillips*, in *Tracking the Vanishing Frogs: An Ecological Mystery* (St. Martin's Press; Penguin paperback)

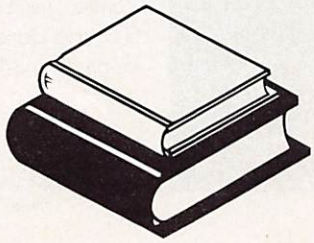
Once, I was a member of a prestigious panel of herpetologists meeting to decide the future of herpetology. There were about eight of us sitting around a conference table. Glancing around, I noticed that about two-thirds of us had a missing digit. Herpetologists cannot resist picking up venomous snakes. Sooner or later, most manage to get bitten, and they often lose part of a digit in the process. I felt right at home in that group, even though I didn't lose mine to a snake (how were they to know?).

—*Eric R. Pianka*, in *The Lizard Man Speaks* (University of Texas Press)

now been crystallized, and a good number of three-dimensional structural models are presented. Schematic representations of the receptors are included as well, creating an immediate impression of the type of signal transduction that is likely to occur (a notable deficiency here concerns the TNF receptor family, for which a number of transducing molecules have emerged since publication). Detailed descriptions of several knockout phenotypes are also presented.

This book meets an obvious need. Many cytokine workers immediately find themselves out of their depth when the conversation (or the impulse of research) turns to a factor with which they have no hands-on experience. For them the *Guidebook* sets forth facts of a solid character that are not likely to change in the near term. This makes the book extremely attractive and well worth buying.

Bruce Beutler
Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75235–9050, USA



Reprints of Books Previously Reviewed

Early Quantum Electrodynamics. A Source Book. Arthur I. Miller. Cambridge University Press, New York, 1995. Paper, $24.95. *Reviewed* **265**, 1605 (1994).

Galileo At Work. His Scientific Biography. Stillman Drake. Dover, New York, 1995. Paper, $14.95. *Reviewed* **206,** 439 (1979).

Polymers. The Origins and Growth of a Science. Herbert Morawetz. Dover, New York, 1995. Paper, $11.95. *Reviewed* **231,** 167 (1986).

The Quark and the Jaguar. Adventures in the Simple and the Complex. Murray Gell-Mann. Freeman, New York, 1995. Paper, $15.95. *Reviewed* **264,** 1480 (1994).

Books Received

Advances in Linear Logic. Jean-Yves Girard, Yves Lafont, and Laurent Regnier, Eds. Cambridge University Press, New York, 1995. viii, 389 pp. Paper, $44.95. London Mathematical Society Lecture Note, 222.

Advances in Mucosal Immunology. Jiri Mestecky *et al.*, Eds. Plenum, New York, 1995. 2 vols. Part A, xliv pp. + pp. 1-698, illus. Part B, iv pp. + pp. 699-1680, illus. $245. Advances in Experimental Medicine and Biology, vol. 371. From a congress, Prague, Aug. 1992.

Biology of the Sialic Acids. Abraham Rosenberg, Ed. Plenum, New York, 1995. xvi, 378 pp., illus. $95.

Biology Takes Form. Animal Morphology and the German Universities, 1800-1900. Lynn K. Nyhart. University of Chicago Press, Chicago, 1995. xiv, 414 pp., illus., + plates. $75 or £59.95; paper, $27.50 or £21.95. Science and Its Conceptual Foundations.

Causal Cognition. A Multidisciplinary Debate. Dan Sperber, David Premack, and Ann James Premack, Eds. Clarendon (Oxford University Press), New York, 1995. xx, 670 pp., illus. $115. Symposia of the Fyssen Foundation. From a symposium, St-Germain-en-Laye, Jan. 1993.

Cell Architecture and Metabolic Channeling. Judit Ovádi. Springer-Verlag, New York, and Landes, Austin, TX, 1995 (distributor, CRC Press, Boca Raton, FL).

viii, 250 pp., illus. $89. Molecular Biology Intelligence Unit.

Disabled Children and Developing Countries. Pam Zinkin and Helen McConachie, Eds. Mac Keith, London, 1995 (U.S. distributor, Cambridge University Press, New York). xii, 238 pp., illus. $59.95. Clinics in Developmental Medicine, no. 136.

Discipline and Experience. The Mathematical Way in the Scientific Revolution. Peter Dear. University of Chicago Press, Chicago, 1995. xiv, 290 pp., illus. $60 or £47.95; paper, $24 or £19.25. Science and Its Conceptual Foundations.

Encyclopedia of Analytical Science. Alan Townshend, Ed. Academic Press, San Diego, 1995. 10 vols. clii, 6059 pp., illus., + plates. $1595.

The Encyclopedia of Snakes. Chris Mattison. Facts on File, New York, 1995. 256 pp., illus. $35 or $C46.95.

Flight Management Systems. The Evolution of Avionics and Navigation Technology. Samuel B. Fishbein. Praeger, Westport, CT, 1995. xxii, 239 pp., illus. $65.

Florida Indians and the Invasion from Europe. Jerald T. Milanich. University Press of Florida, Gainesville, 1995. xx, 290 pp., illus. $29.95.

Fluvial Processes in Geomorphology. Luna B. Leopold, M. Gordon Wolman, and John P. Miller. Dover, New York, 1995. xiv, 522 pp., illus. Paper, $17.95. Reprint, 1964 ed.

Global Warming. The Truth Behind the Myth. Michael L. Parsons. Insight Books (Plenum), New York, 1995. xvi, 271 pp., illus. $27.95.

"Glucokinase." Its Regulation and Role in Liver Metabolism. María Luz Cárdenas. Springer-Verlag, New York, and Landes, Austin, TX, 1995 (distributor, CRC Press, Boca Raton, FL). xii, 210 pp., illus. $89. Molecular Biology Intelligence Unit.

Hidden Order. How Adaptation Builds Complexity. John H. Holland. Addison-Wesley, Reading, MA, 1995. xxii, 185 pp., illus. $24 or $C33.

High Temperature Superconductivity. Current Results and Novel Mechanisms. K. P. Sinha and S. L. Kakani. Nova, Commack, NY, 1995. x, 270 pp., illus. $93.

Interfacial Phenomena in Drug Delivery and Targeting. Graham Buckton. Harwood, Langhorne, PA, 1995 (distributor, International Publishers Distributor, Brooklyn, NY). xxiv, 289 pp., illus. $95 or £62 or ECU 79. Drug Targeting and Delivery, vol. 5.

Interleukin-6-Type Cytokines. Andrezej Mackiewicz, Aleksander Koj, and Pravin B. Sehgal, Eds. New York Academy of Sciences, New York, 1995. xvi, 522 pp., illus. $150. Annals of the New York Academy of Sciences, vol. 762. From a conference, Poznan, Poland, June 1994.

Marine Ecological Processes. Ivan Valiela. 2nd ed. Springer-Verlag, New York, 1995. xiv, 686 pp., illus. $54.95.

The Mathematics of Generalization. The Proceedings of the SFI/CNLS Workshop on Formal Approaches to Supervised Learning. David H. Wolpert, Ed. Addison-Wesley, Reading, MA, 1995. xviii, 441 pp. $55.95; paper, $31.95. Santa Fe Institute Studies in the Sciences of Complexity, Proceedings, vol. 20.

New Approaches to Speciation in the Fossil Record. Douglas H. Erwin and Robert L. Anstey, Ed. Columbia University Press, New York, 1995. xii, 342 pp., illus. $45 or £34. Based on a meeting, 1992.

New Frontiers in Medical Device Technology. Arye Rosen and Harel D. Rosen, Eds. Wiley, New York, 1995. xii, 364 pp., illus. $69.95. Wiley Series in Microwave and Optical Engineering.

Oxidative Stress, Lipoproteins and Cardiovascular Dysfunction. C. Rice-Evans and K. R. Bruckdorfer, Eds. Portland, London, 1995 (U.S. distributor, Ashgate, Brookfield, VT). xvi, 184 pp., illus. $96 or £60. Portland Press Research Monographs, 7.

Palaeozoic Palaeobotany of Great Britain. C. J. Cleal and B. A. Thomas. Chapman and Hall, New York, 1995. xii, 295 pp., illus. $129.95.

Paleobotany. Plants of the Past, Their Evolution, Paleoenvironment and Application in Exploration of Fossil Fuels. Shripad N. Agashe. Science Publishers, Lebanon, NH, 1995. x, 359 pp., illus. $55.

Questioning Chemotherapy. Ralph W. Moss. Equinox, Brooklyn, NY, 1995. ii, 214 pp. Paper, $19.95 or $C25.95.

Radiogenic Isotope Geology. Alan P. Dickin. Cambridge University Press, New York, 1995. xvi, 452 pp., illus. $89.95.

Rapid Freezing, Freeze Fracture, and Deep Etching. Nicholas J. Severs and David M. Shotton, Eds. Wiley-Liss, New York, 1995. xii, 372 pp., illus. $125. Techniques in Modern Biomedical Microscopy.

Scientific Integrity. An Introductory Text with Cases. Francis L. Macrina. ASM Press, Washington, DC, 1995. xxii, 283 pp. Paper, $24.95.

Topology. An Introduction to the Point-Set and Algebraic Areas. Donald W. Kahn. Dover, New York, 1995. viii, 217 pp., illus. Paper, $7.95. Reprint, 1975 ed.

Work and Technology in Higher Education. The Social Construction of Academic Computing. Mark A. Shields, Eds. Erlbaum, Hillsdale, NJ, 1995. viii, 198 pp., illus. $49.95; paper, $19.95. Technology and Education.

Publishers' Addresses

Below is information about how to direct orders for books reviewed in this issue. A fuller list of addresses of publishers represented in *Science* appears in the issue of 26 May 1995, page 1220.

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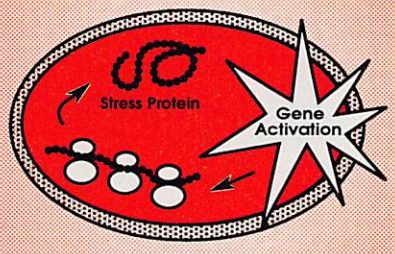

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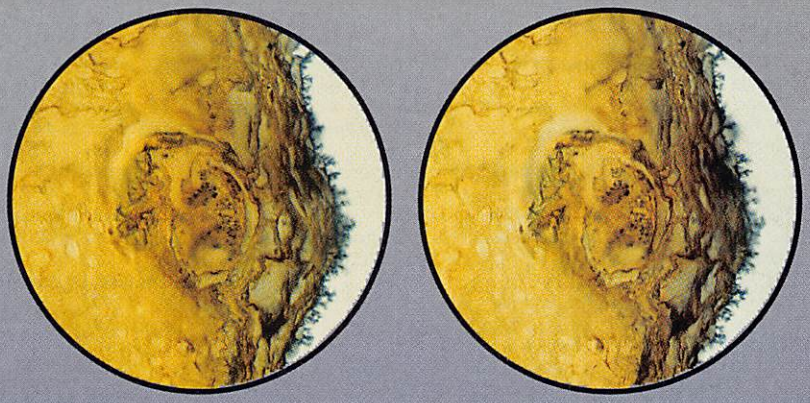

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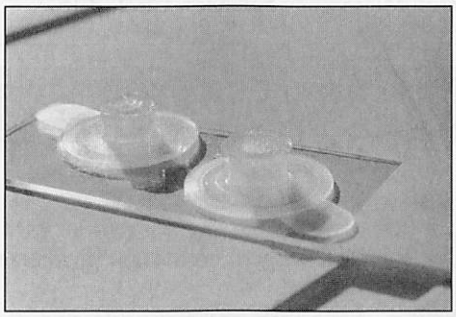
Microscope Slide Reaction Chamber

Gene Cone is a reaction chamber to prevent evaporation of amplification reagents thermocycling on a microscope slide. The user simply peels and sticks the self-adhesive chamber over a cell specimen or tissue section. The chamber allows amplification of RNA or DNA targets on a microscope slide just as solution reactions are done in tubes. Most of the amplified material remains localized for detection within the cells. The Slide



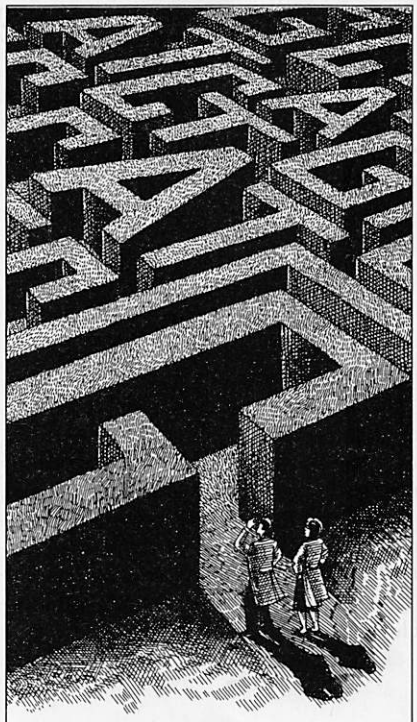
Adaptor kit combines everything needed to thermocycle slide specimens: heating platen and spring-pressure platform, 24 Gene Cone chambers, and 30 temperature monitors. **Gene Tec. Circle 143.**

■

Drawing and Diagramming Software

Drag 'n Draw for Windows 95 provides a quick and easy way to create designs and diagrams without the steep learning curve associated with most drawing applications. This new 32-bit version upgrades a recently launched 16-bit version. The program includes more than 1500 pre-drawn objects in symbol libraries so users do not have to spend time creating objects. Users simply select

Newly offered instrumentation, apparatus, and laboratory materials of interest to researchers in all disciplines in academic, industrial, and government organizations are featured in this space. Emphasis is given to purpose, chief characteristics, and availability of products and materials. Endorsement by *Science* or AAAS is not implied. Additional information may be obtained from the manufacturers or suppliers named by circling the appropriate number on the Readers' Service Card and placing it in a mailbox. Postage is free.



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objects from libraries and drop them into a drawing. The version is packaged with both the Windows 3.1 version and the Windows 95 version in the same box. **DeltaPoint. Circle 144.**

■

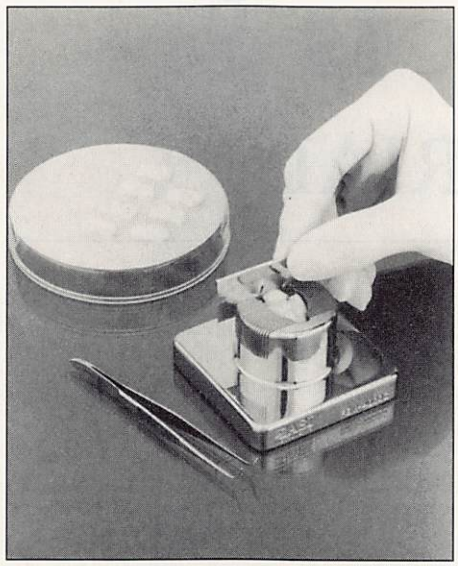
Messenger RNA Detection and Quantitation Kit

NorthernMax is a kit containing all the reagents and solutions necessary for detection and quantitation of mRNA. The kit is optimized for use with Ambion's BrightStar chemiluminescent labeling and detection system, generating high signal-to-noise ratios and low background with sensitivities as good or better than radioisotopic analysis. Results can be available in minutes. The kit also can be used in isotopic format. **Ambion. Circle 145.**

■

Tissue and Rodent Brain Matrices

Tissue and Rodent Brain Matrices can aid the researcher in the freehand dissection of a wide variety of different tissues and rodent brains. They can help researchers repeatedly slice fresh tissue and brain in intervals as small as 1 mm. The matrices are made from high-grade aluminum for rapid temperature equilibration, ease of cleaning, and sterilization. Applications include biochemical pharmacology, anatomy, and neurophysiology. Standard matrices available include gerbil, mouse, rat, guinea pig, ferret, rabbit, hamster, monkey, dog, cat, turkey, chicken, and canary. Heart matrices are also available. **Electron Microscopy Sciences. Circle 146.**



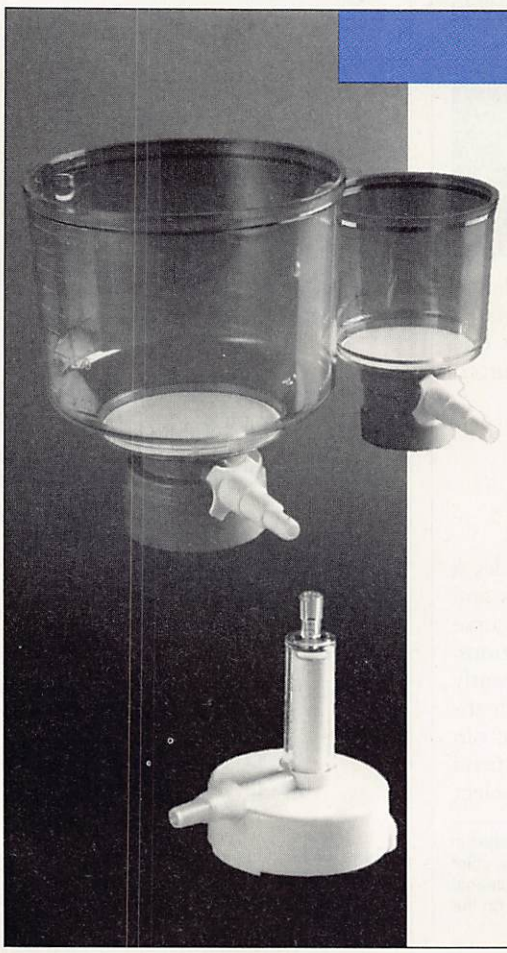
Literature

Chiral Applications Guide is a brochure that describes the capabilities of a capillary electrophoresis system for enantiomeric separations. **Beckman Instruments. Circle 147.**

Orbital Shakers describes a line that includes a new model designed to be used on a lab bench, in a reach-in incubator, or in an environmentally controlled room. Also highlighted are additional platforms and accessories. **Forma Scientific. Circle 148.**

The Ultimate Microwave Digestion Systems showcases a line of microwave instruments, accessories, and software. It covers the latest advances in high-pressure/closed vessel digestions, including improved instruments, vessels capable of pressures up to 1000 psi and temperatures to 260°C, and advanced fiber optic temperature sensing. **CEM Corp. Circle 149.**

Understanding Chemifluorescence is a full-color guide to a nonisotopic labeling and detection method for proteins and nucleic acids. The guide presents information on the principles of chemifluorescence chemistry, the advantages over other detection methods, and the range of applications. **Molecular Dynamics. Circle 150.**



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POSITIONS OPEN

STATE UNIVERSITY OF NEW YORK AT BUFFALO DEPARTMENT OF BIOCHEMISTRY

The Department of Biochemistry in the School of Medicine and Biomedical Sciences invites applications for a faculty opening at the **ASSISTANT, ASSOCIATE,** or **FULL PROFESSOR** level for a twelve-month, state-funded, tenure-track position. Preference will be given to established investigators with a nationally recognized research program supported by major extramural funding. We will also consider especially outstanding new investigators who have demonstrated exceptional research ability and promise for contributing to interdisciplinary training programs for graduate and professional students. Applicants should have a Ph.D. or M.D. degree. Applicants from all areas of biochemistry and molecular biology are encouraged to apply, but we are especially seeking candidates who have expertise in molecular mechanisms of regulation in eukaryotic systems. The Medical School enjoys interdisciplinary interactions with faculty throughout the University. A major structural biology program is underway and recently a new 120,000 square-foot research building has been opened. These developments present an uncommon opportunity for determined scientists to advance their research goals in an exciting environment. Send a complete curriculum vitae, a statement of research interests and future plans, reprints of recent publications and the names, addresses and FAX numbers of at least three referees to: **Chairman, Faculty Search Committee, Biochemistry Department, 140 Farber Hall, State University of New York, 3435 Main Street, Buffalo, NY 14214-3000.** Screening will begin immediately. To be assured of full consideration, applications should be received by February 1, 1996. *The State University of New York is an Affirmative Action/Equal Opportunity Employer.*

ASSISTANT/ASSOCIATE PROFESSOR PHARMACOLOGY

The Ohio State University College of Pharmacy, Division of Pharmacology, invites applications for a tenure-track position at the **ASSISTANT** or **ASSOCIATE PROFESSOR** level. Candidates should have a Ph.D. in pharmacology, toxicology, or related field. Areas of high priority include biochemical, molecular, or cellular studies involved in the pharmacological or toxicological actions of drugs. Applicants at the Assistant Professor level should have significant postdoctoral research experience and demonstrated excellence in conducting research. This position offers excellent opportunities for an individual committed to quality research and teaching and willing to work in a collaborative environment within our health science community. Review of applications will begin January 1, 1996 with the position starting no later than September 1, 1996. A curriculum vitae, a description of research interests, and the names and addresses of at least three persons from whom letters of recommendation can be solicited should be sent to: **Dennis B. McKay, Ph.D., Chair of Search Committee, Division of Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH 43210.** *The Ohio State University is an Equal Opportunity/Affirmative Action Employer. Women, minorities, Vietnam-era veterans, disabled veterans and individuals with disabilities are encouraged to apply.*

Tulane University is seeking to fill a vacant faculty position at the **TENURE-TRACK ASSISTANT PROFESSOR** level. It is possible that a candidate with exceptional qualifications may be considered for a position at a higher level. We are seeking a person with a Ph.D. at the time of appointment that can demonstrate high standards of both teaching and research, and who emphasizes applications of geology to environmental problems. Such a person may have a research field in any specialty, however, strong consideration will be given to those candidates that specialize in fields such as sedimentary geology, geohydrology, geomorphology, remote sensing, geochemistry, or geophysics, and can interact with researchers and students in a developing interdisciplinary environmental science program at Tulane. Applicants should send a letter of application, statement of research and teaching interests, current curriculum vitae, and the names, addresses, and telephone numbers of three references to: **Stephen A. Nelson, Department of Geology, Tulane University, New Orleans, LA 70118. Email: snelson@mailhost.tcs.tulane.edu.** The search will remain open until a qualified applicant is found. *Tulane University is an Affirmative Action/Equal Opportunity Employer. Women and minorities are encouraged to apply.*

POSITIONS OPEN

DEVELOPMENTAL/EVOLUTIONARY BIOLOGIST ASSISTANT PROFESSOR

The Biology Department of St. Lawrence University invites applications for a tenure-track position at the **ASSISTANT PROFESSOR** level to begin in the fall of 1996. We are seeking a developmental/evolutionary biologist with a demonstrated competence to teach laboratory courses in developmental biology and genetics. The ability to teach comparative vertebrate morphology, evolution, and other courses in one's area of expertise is also desired. The typical teaching load will be two lab courses per semester. A Ph.D. is required along with a commitment to teaching at the undergraduate liberal arts level. The successful candidate will be expected to develop a strong research program that would involve undergraduate students and to teach in the University's interdisciplinary program for first year students prior to tenure evaluation. Experience using computer-based multimedia in teaching would also be desirable. Candidates should submit a curriculum vitae, a statement of teaching philosophy and interest, a statement of current and future research interests, and have three letters of recommendation forwarded to: **Dr. T. Budd, Biology Department, St. Lawrence University, Romoda Drive, Canton, NY 13617.** Applications should be postmarked no later than January 26, 1996. *St. Lawrence University, a private, nonsectarian undergraduate university, is an Affirmative Action/Equal Employment Opportunity Employer. Women, minorities, veterans, and persons with disabilities are encouraged to apply.*

CELL CYCLE REGULATION
Cornell University Medical College

The Department of Cell Biology and Anatomy invites applications for a tenure-track position at the **ASSISTANT** or **ASSOCIATE PROFESSOR** level. Candidates whose analysis of cell cycle regulation involves a genetic approach, addresses networks of protein phosphorylation, or relates to development or oncogenesis are of particular interest. The successful candidate will join an interactive group of faculty with broad research interests in cell, developmental, and molecular biology. Candidates will be expected to establish a strong, well-funded research program and to contribute to graduate and medical education. Interested candidates should submit a curriculum vitae, names and addresses of three references, and a description of current and future research interests by January 31, 1996 to: **Paula Traktman, Ph.D., Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.** *Equal Employment Opportunity/Affirmative Action/Minorities/Female/Disabled/Veteran.*

Morehead State University invites applications for a tenure-track position as **ASSISTANT PROFESSOR** of Biology/Botanist beginning August 1996. Responsibilities: teach courses in plant anatomy, plant taxonomy, plant morphology, general botany, field botany, and general biology; enhance a greater than eleven-thousand herbarium; manage a greenhouse; take an active role in both biology (B.S. and M.S.) and environmental science (B.S.) programs. Job requires extensive knowledge of diverse Central/Southern Appalachian bryophytes. Qualifications: Ph.D. in biological science. Strong communication skills. Evidence of potential for successful teaching, research, and service. To ensure consideration, submit letter of application, curriculum vitae, statements of teaching philosophy and research interests, and three letters of reference by January 15, 1996, to: **Office of Human Resources, Attn: Botanist, Morehead State University, HM 101, Morehead, KY 40351.** *Morehead State University is an Affirmative Action/Equal Opportunity Employer.*

The Division of Virology, which is affiliated with the Division of Infectious Diseases at the University of Washington, is recruiting a full-time faculty member at the **ASSISTANT PROFESSOR** level. Applicants must have extensive laboratory-based research training in viral-pathogenesis and immunology of medical interest. Preference will be given to those involved in sexually transmitted viruses or viruses of the immunocompromised host. Applicants should be ABIM certified in internal medicine and infectious diseases and will participate in the clinical activities of the division. Send curriculum vitae and three letters of reference to: **Lawrence Corey, M.D., Division of Virology, Department of Laboratory Medicine/Medicine, 1200 12th Avenue South, Room 9301, Seattle, WA 98144.** *The University of Washington is an Affirmative Action/Equal Opportunity Employer.* The closing date for applications is January 31, 1996.

POSITIONS OPEN

PLANT ECOLOGIST
GRINNELL COLLEGE

Grinnell College invites applications for a tenure-track appointment beginning August 1996, in plant ecology at the **ASSISTANT PROFESSOR** rank. We seek candidates with proficiency in quantitative methods of the discipline and a commitment to field research with the ability to teach an advanced course in ecology using our field station and laboratory, an introductory biology course in evolution and ecology, and a general education course for non-majors. Grinnell College is seeking candidates with postdoctoral research experience who plan an active research program involving undergraduates. Start-up funds, excellent facilities, and continuing support for research are available. In their applications, candidates should address their interests in teaching in an undergraduate, liberal-arts environment that emphasizes close faculty-student interaction. Send curriculum vitae, three letters of recommendation, a statement of teaching and research interests, and copies of all transcripts to: **Professor Bruce Voyles (voyles@ac.grin.edu), Ecology Search Committee, Department of Biology, Grinnell College, Grinnell, IA 50112-0806. Telephone: 515-269-3047.** To ensure full consideration, applications must be complete by January 26, 1996. *Grinnell College is an Equal Opportunity/Affirmative Action Employer and especially seeks women and minority candidates.*

ASSISTANT PROFESSOR
NEUROBIOLOGY
DePaul University

DePaul University seeks an individual with a strong commitment to undergraduate education for this **TENURE-TRACK** position. In this newly created role you will teach graduate (M.S.)/advanced-undergraduate neurobiology, a specialty course, introductory biology, and possibly anatomy. We require a Ph.D. and active research with start-up funds available. Previous teaching experience desirable.

All applications must include a curriculum vitae with letter of interest, three letters of reference, a statement of educational philosophy, and a list of equipment/supply needs with cost estimates by item/category. Send or FAX to: **Dr. Sidney L. Beck, Chair, Department of Biological Sciences, DePaul University, 1036 West Belden Avenue, Chicago, IL 60614. FAX: 312-325-7596; Email: sbeck@condor.depaul.edu.** *DePaul University, the employer of choice, is committed to diversity and equality in education and employment.*

ASSISTANT PROFESSOR, Conservation Biology. Department of Biological Sciences, Florida Institute of Technology. Permanent nine-month position beginning August 1996. Ph.D. and postdoctoral research required. GIS experience preferred. Candidates must have research experience in applied conservation problems related to reserve design, protected species management, and marine conservation issues. Duties: developing a strong, funded research program; advising graduate students for the M.S. and Ph.D.; teaching undergraduate and graduate courses in ecology, conservation and evolutionary biology and others related to candidate's research interests. Send curriculum vitae, plans for research and teaching, selected reprints, and names of three references to: **Dr. G. N. Wells, Head, Department of Biological Sciences, Florida Institute of Technology, 150 West University Boulevard, Melbourne, FL 32901. Telephone: 407-768-8000, ext. 8034; Email: gwells@fit.edu.** Position pending funding. *Affirmative Action/Equal Opportunity Employer.*

ENVIRONMENTAL STUDIES
BIOLOGY
ELON COLLEGE

Applicants are invited for **ASSISTANT/ASSOCIATE PROFESSOR** of biology and **COORDINATOR** of environmental studies. Master's degree in biology and Ph.D. in environmental studies or related area required. A private, liberal arts college with 3500 students, Elon is located between Greensboro and Chapel Hill, North Carolina. Send letter of application, curriculum vitae, graduate transcripts, statement of teaching and research goals, and at least three letters of reference to: **Dr. Rosalind Reichard, Associate Dean of Academic Affairs, 2163 Campus Box, Elon College, NC 27244.** Applications should be received by January 19, 1996. *Minority and women candidates are encouraged to apply. Elon is an Equal Employment Opportunity Institution.*

POSITIONS OPEN

FACULTY POSITIONS
IN
MEMBRANE/CELL PHYSIOLOGY

The Department of Physiology of the Medical College of Pennsylvania and Hahnemann University invites applications for full-time, tenure-track faculty positions at the rank of **ASSISTANT PROFESSOR, ASSOCIATE PROFESSOR** or **PROFESSOR.** Applicants must have a record of independent research in an area of membrane or cell physiology. Future areas targeted for expansion include epithelial and cardiovascular membrane structure/function. Appointees will join a Department that was initiated in 1992 and is built around the theme of membrane and cell physiology. Such a cohesive Departmental theme ensures excellent opportunities for interactions and collaboration. The Department occupies space in a recently renovated building located in a very pleasant residential area of Philadelphia. Salaries and start-up packages are competitive. In addition to establishing and maintaining a competitive research program, successful candidates will be expected to participate in teaching in a medical physiology course. Applications will be accepted until March 1, 1996. Send a curriculum vitae, a brief description of research interests and plans, and the names, addresses, and telephone numbers of at least three references to: **Dr. John M. Russell, Department of Physiology, Medical College of Pennsylvania and Hahnemann University, 2900 Queen Lane, Philadelphia, PA 19129. Email: russell@medcolpa.edu.**

BEHAVIORAL/COGNITIVE
NEUROSCIENTIST

The Department of Brain and Cognitive Sciences in the University of Rochester seeks an **ASSISTANT PROFESSOR** for a tenure-track position. We are interested in persons working within the broad domain of brain and behavioral plasticity. Individuals whose research concerns neural mechanisms of learning and memory, or development/reorganization in perceptual or motor systems, are especially encouraged to apply. The successful candidate will be a key member of a group employing neurobiological, computational, and behavioral approaches to learning and developmental plasticity. Potential applicants can learn more about the Department and its programs from our web site: **http://www.bcs.rochester.edu/bcs/.** Applicants should submit a curriculum vitae, a brief statement of research interests, and the names of three referees to: **Search Committee Chair, Department of Brain and Cognitive Sciences, University of Rochester, Rochester, NY 14627.** Applications will be accepted until 1 February 1996, or until a suitable candidate is found. *An Affirmative Action/Equal Opportunity Employer.*

ASSISTANT PROFESSOR
BIOLOGY
Temple University

The Biology Department at Temple University is seeking from one to three **TENURE-TRACK** faculty members in physiology/ecology at the assistant professor level. Appointees would be expected to develop active research programs and to demonstrate excellence in graduate and undergraduate teaching. The department is broadly based, with 23 faculty members and an established graduate program leading to the M.A. and Ph.D. degrees. Candidates should submit curriculum vitae, research plans, descriptions of teaching experience and philosophy, and ask three referees to send letters of recommendation to: **Dr. Joel B. Sheffield, Chair, Department of Biology, Temple University, Philadelphia, PA 19122.** We will begin to review applications on February 1, 1996. For additional information **Telephone: 215-204-8854, FAX: 215-204-6646,** or review our web site: **http://blue.temple.edu/biology.** *An Equal Opportunity/Affirmative Action Employer.*

The Department of Psychology, Northwestern University, invites applications for a **TENURE-TRACK ASSISTANT PROFESSOR** position in the area of cognitive neuroscience (including computational modeling) to begin September 1996 or later. Qualifications include outstanding research and teaching ability. Interested candidates should send a statement of research interests, curriculum vitae, copies of recent publications, and at least three letters of recommendation to: **The Cognitive Neuroscience Search Committee, Department of Psychology, Northwestern University, Evanston, IL 60208-2710, USA.** *Northwestern University is an Affirmative Action/Equal Opportunity Employer.*

POSITIONS OPEN

ASSISTANT PROFESSOR
BIOLOGICAL SCIENCE

Applications are invited for a tenure-track, **ASSISTANT PROFESSOR** position in the general area of environmental biology. The successful candidate will be expected to be committed to undergraduate education and to develop an active, extramurally funded research program that supports Master's and Doctoral level graduate students. Preference will be given to applicants with research expertise in the areas of toxicology, aquatic ecology, or developmental biology related to environmental concerns although other environmental-related research areas will be considered.

Michigan Technological University promotes the formation of interdisciplinary research programs and numerous opportunities for research collaborations are available in conjunction with faculty in the Environmental Engineering Center, the EPA Center for Clean Industrial and Treatment Technologies, and the Lake Superior Research group. Construction of a $50 million Environmental Sciences and Engineering complex that houses the Department of Biological Sciences will begin shortly.

Review of applications will commence February 1, 1996 and will continue until the position is filled. Send applications, consisting of curriculum vitae, a brief statement of teaching philosophy and research goals, copies of recent publications, and a list of three references, to: **Search Committee, Department of Biological Sciences, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931-1295.** *Michigan Technological University is an Affirmative Action/Equal Opportunity Employer/Educational Institution.*

FACULTY POSITION
BIOLOGICAL CHEMISTRY

The Department of Chemistry at the University of North Carolina at Chapel Hill invites applications for a tenure-track position in biological chemistry. The successful applicant must have a Ph.D., postdoctoral experience, a strong record of research, and the ability to teach biological chemistry effectively at the undergraduate and graduate levels. Preference will be given to individuals who can establish a strong research program in biological chemistry and interact enthusiastically with other chemists, and whose appointment would be at the rank of **ASSISTANT PROFESSOR.** Review of the applications will begin December 15, 1995. Send a curriculum vitae, a brief research plan, a statement of teaching interests, and four letters of recommendation to: **Dr. Nancy L. Thompson, Department of Chemistry, CB 3290 University of North Carolina, Chapel Hill, NC 27599-3290.** *The University of North Carolina is an Equal Opportunity/Affirmative Action Employer.*

FACULTY POSITION
DEPARTMENT OF PATHOLOGY
University of Arkansas for Medical Sciences

Applications or nominations are invited for a faculty position in the Department of Pathology, Division of Pediatric Pathology, at the level of **ASSISTANT PROFESSOR.** Applicants should have an established track record of excellence in original research in tumor cell biology as evidenced by their training and publications. Ph.D. level and postgraduate training and experience in molecular genetics is required. Candidates should submit their curriculum vitae to: **David M. Parham, M.D., Chief of Pediatric Pathology, Slot 820, Arkansas Children's Hospital, 800 Marshall Street, Little Rock, AR 72202.** *An Affirmative Action/Equal Opportunity Employer.*

ASSISTANT PROFESSOR—RESEARCH
BIOCHEMISTRY/BIOLOGY OF BONE

The Department of Orthopedic Surgery at the SUNY Health Science Center (HSC) at Syracuse has an immediate opening for a Senior Research Scientist. Primary responsibilities include developing a research program in the area of bone biochemistry or biology as it relates to orthopaedic implants. Ph.D. in biochemistry or biological sciences, experience with biology/biochemistry of bone, and previous animal experience is required. Send letter, curriculum vitae, and names and telephone numbers of three references to: **Kenneth A. Mann, Ph.D., Department of Orthopedic Surgery, 750 East Adams Street, Syracuse, NY 13210.** *SUNY-HSC is an Affirmative Action/Equal Employment Opportunity/ADA Employer, committed to excellence through diversity.*

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Overlapping images: Howard Sochurek, Genes/DNA, 1992 and Indian cave paintings, Southwestern U.S.

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POSITIONS OPEN

EXPERIMENTAL PATHOLOGIST

The Department of Pathobiology at the University of Florida College of Veterinary Medicine has a tenure-track position for an experimental pathologist at the rank of **ASSOCIATE/ASSISTANT PROFESSOR**. Applicants should have D.V.M. and Ph.D. degrees or equivalents and a record of accomplishment in research. Responsibilities will include development of a competitive research program, teaching general pathology to veterinary students, and graduate instruction. ACVP board-certification is desirable, but not required. The Department has excellent research facilities within a dynamic Health Science Center complex and strong, extramurally funded research programs in molecular parasitology, viral and mycoplasmal disease pathogenesis, vaccine development, and wildlife diseases. Applicants should submit a letter outlining professional goals, a curriculum vitae, and a list of three professional referees to: **Dr. William L. Castleman, Chairman, Department of Pathobiology, College of Veterinary Medicine, P.O. Box 110880, University of Florida, Gainesville, FL 32611-0880.** Department World Wide Web Page: **http://www.vetmed.ufl.edu/path/path.htm**. Review of applications will begin after January 1, 1996 and will continue until the position is filled. The position will start on or after July 1, 1996.

The University of Florida is an Equal Opportunity/Affirmative Action Institution.

ASSISTANT PROFESSOR/BIOCHEMIST

The University of Southern Indiana invites applications for a **TENURE-TRACK** position beginning fall 1996. Responsibilities will include teaching biochemistry, advanced biology courses appropriate to the candidate's expertise, and introductory-level courses in both chemistry and biology. Ph.D. required. Evidence of strong teaching and potential for scholarly research/grant writing required. The University is committed to excellence in teaching, scholarship and professional activity, and service to the University and the community. Biochemists with interest or experience in environmental areas are encouraged to apply. Application deadline is January 15, 1996, or until position is filled. Submit letter of application, curriculum vitae, and names and addresses of three professional references to: **Dr. Marie Hankins, Chair, Department of Chemistry, University of Southern Indiana, 8600 University Boulevard, Evansville, IN 47712.** *Affirmative Action/Equal Opportunity Employer.*

FOOD SAFETY/THERMAL PROCESSING

ASSISTANT PROFESSOR. The Department of Nutrition and Food Science, University of Maryland, invites applications for a nine and a half-month tenure-track research/teaching position. Applicants must have a Ph.D. in food science or related discipline; postdoctoral research or relevant industrial experience in microbial food safety and thermal processing is preferred. Responsibilities include 1) establishing an independent research program on heat preservation/processing including microbial survival and the application of molecular biological methods for pathogen detection in food; and 2) teaching undergraduate and graduate courses in thermal food processing and food safety. Send curriculum vitae, statement of research and teaching interests, academic transcripts, and addresses of three references to: **Dr. Donald V. Schlimme, Department of Nutrition and Food Science, Room 3304 Marie Mount Hall, The University of Maryland, College Park, MD 20742-7521.** Deadline for application is March 1, 1996 or until position is filled. *The University of Maryland is an Equal Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.*

RESEARCH ASSISTANT PROFESSOR or **POSTDOCTORAL** position in the Department of Pediatrics, Duke University Medical Center. This is a position working in the laboratory of **Dr. Michael M. Frank**, Chairman, Department of Pediatrics. This well-funded laboratory explores the mechanism of action of the complement system proteins and the biochemistry of mediators of immune damage. Candidates should submit a cover letter, curriculum vitae and bibliography, and the names of three references to: **Dr. Frank, Box 3352, Duke University Medical Center, Durham, NC 27710.** *Duke University is an Equal Opportunity/Affirmative Action Employer.*

MAMMALIAN OR AVIAN PHYSIOLOGIST

Tenure-track, **ASSISTANT PROFESSOR** position anticipated for fall 1996, in Biological Sciences at the University of New Orleans (UNO); Ph.D. required and postdoctoral research experience in physiological adaptations of mammals or birds preferred. The Department is affiliated with the Audubon Institute's Center for Research of Endangered Species providing research opportunities involving threatened and endangered mammals and birds. Initially, duties include developing an upper-level animal physiology laboratory, and later, participating in upper-level physiology courses and Introductory Biology. Our twenty-four Graduate Faculty are expected to seek outside funding and engage in research, direct graduate student research, and teach courses. UNO (approximately 16,000 students) is in the LSU System on a campus overlooking Lake Pontchartrain. A statement of research interests, recent reprints, curriculum vitae, and three letters of recommendation should be sent by February 23, 1996 to: **Dr. Britt Bromberg, Search Committee, Biological Sciences, University of New Orleans, New Orleans, LA 70148. Email: BBBBS@UNO.EDU.** *UNO is an Equal Opportunity/Affirmative Action Employer.*

ENVIRONMENTAL STUDIES/BIOLOGY

ASSISTANT PROFESSOR. Randolph-Macon College invites applications for an entry-level, nine-month tenure-track position in environmental studies/biology beginning in August 1996. Candidates should have a Ph.D. in environmental studies or a related field, with a specialty in wetlands study, aquatic biology, wildlife biology, or resource management. Candidates must be committed to undergraduate teaching in the liberal arts tradition, research involving undergraduate students, and development of the environmental studies program. Teaching responsibilities will include the introductory environmental studies sequence, and existing or new courses in environmental studies or biology, especially field biology. Candidates should submit curriculum vitae, a statement of teaching and research goals, and three letters of reference by January 17, 1996 to: **C. Barry Knisley, Chair, Environmental Search, Randolph-Macon College, Ashland, VA 23005.** *Randolph-Macon College is an Equal Opportunity/Affirmative Action Employer.*

AQUATIC BIOLOGIST

Lake Superior State University is seeking a tenure-track aquatic biologist **FACULTY MEMBER.** The academic program in biology is undergraduate. Teaching load will be 12 contract hours per semester (1 per lecture 2/3 per lab). Duties will include advisement, committee work, and supervision of senior thesis projects. Opportunity to develop a course in area of expertise and opportunity for research in conjunction with the Lake Superior State University Aquatic Research Lab.

Qualifications: A Ph.D. in aquatic biology, interest and excellence in undergraduate teaching and advising, and the ability to lead students in undergraduate research. Experience in aquatic ecotoxicology and federal or state agency experience is preferred.

Complete applications consist of a letter of application addressing the above qualifications, curriculum vitae, unofficial transcripts, and three letters of reference to: **Aquatic Biology, Office of Employee Relations, Lake Superior State University, Administration Building, Sault Ste. Marie, MI 49783. FAX: 906-635-2111.** For further information, contact **Karel Rogers, Telephone: 906-635-2266; Email: krogers@lakers.lssu.edu** or **www.lssu.edu.**

Application deadline: January 15, 1996.

An Equal Employment Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.

FACULTY FOR EUROPE AND ASIA

The University of Maryland anticipates openings for **FACULTY** to teach undergraduate science courses on U.S. military bases overseas. Requirements include the Ph.D. or two Master's degrees, recent college teaching experience, *U.S. citizenship*, and competence to teach in two of the following fields: astronomy, biology, chemistry, computer studies, geology, mathematics, and physics. Renewable annual appointments begin August 1996. Frequent relocation and the cost of schooling make these positions difficult for those with children. Send résumé to: **Dr. Rosemary Hoffmann, University of Maryland University College, College Park, MD 20742-1642.** *Affirmative Action/Equal Employment Opportunity.*

TWO FACULTY POSITIONS
DEPARTMENT OF BIOLOGY
TEXAS A&M UNIVERSITY

Applications are invited for two tenure-track positions at the **ASSISTANT PROFESSOR** level in the Department of Biology with a joint appointment in the Center for Advanced Invertebrate Molecular Sciences. We seek outstanding individuals who use cellular, molecular, and genetic approaches to address fundamental questions using model invertebrate systems. Preference will be given to candidates working in the areas of Drosophila development and invertebrate immunity or receptor biology. Successful candidates will be expected to establish a vigorous research program and to participate in undergraduate and graduate teaching. Interested individuals should send curriculum vitae and a statement of research and teaching interests, and should arrange to have three letters of reference sent to: **Invertebrate Molecular Biology Search Committee, Department of Biology, Texas A&M University, College Station, TX 77843-3258. FAX: 409-845-2891.** Deadline: January 22, 1996. *Texas A&M University is an Equal Opportunity/Affirmative Action Employer.*

MOLECULAR BIOLOGIST
FORDHAM UNIVERSITY

Individuals are invited to apply for a tenure-track position at the **ASSOCIATE PROFESSOR** level. The department has an active research program and provides excellent physical facilities, state-of-the-art equipment, a stimulating research environment, start-up funds, and competitive salaries and benefits. Candidates should possess expertise in the area of cell/molecular biology and have an ongoing grant-supported research effort underway. The appointee will be expected to establish an active research program and participate in teaching at the graduate and undergraduate levels. Please submit a curriculum vitae and the names and addresses of three references by 15 January 1996 to: **Dr. Berish Y. Rubin, Chairman, Department of Biological Sciences, Fordham University, Bronx, NY 10458.** *Fordham University is an Affirmative Action/Equal Opportunity Employer.*

LOYOLA UNIVERSITY CHICAGO
STRITCH SCHOOL OF MEDICINE
FACULTY POSITION
Department of Microbiology and Immunology

The Department of Microbiology & Immunology (Chair: **Dr. Katherine L. Knight**) invites applications for a **TENURE-TRACK** appointment. The candidate is expected to develop an independent, extramurally funded research program using mammalian, yeast, or prokaryotic models to study molecular aspects of immunology, virology, or bacteriology. Excellent new facilities and attractive start-up funds are available. Applicants should send a curriculum vitae, a concise statement of their present and future research interests, and names and addresses of three references to: **Dr. Hans-Martin Jäck, Chair, Search Committee, Department of Microbiology and Immunology, Loyola University Chicago, 2160 South First Avenue, Maywood, IL 60153.**

Environmental Chemistry **FACULTY POSITION**, tenure-track, any rank, in the Chemistry Department, State University of New York–College of Environmental Science and Forestry (SUNY-CESF). A Ph.D. in chemistry or related discipline, with emphasis in environmental chemistry, is required. Strength in physical chemistry and postdoctoral experience are preferred. The successful candidate will be expected to develop a strong, extramurally funded research program and teach effectively at the undergraduate and graduate level. The department has 13 faculty members and offers B.S., M.S. and Ph.D. degrees with specializations in biochemistry, environmental, natural products and polymer chemistry. Information on the Department and its new building can be found at **http://www.esf.edu/**. The position is available starting August 15, 1996. To apply, submit a detailed curriculum vitae, research plans, and three letters of recommendation to: **John P. Hassett, Faculty of Chemistry, SUNY-CESF, 1 Forestry Drive, Syracuse, NY 13210-2778.** Deadline is January 15, 1996. *SUNY-CESF is an Equal Opportunity/Affirmative Action Employer. Women and minorities are especially encouraged to apply.*

ANNOUNCING THE UNCF•MERCK POSTDOCTORAL SCIENCE RESEARCH FELLOWSHIPS

Ten $55,000 awards will be made in 1996. Each award consists of

- **FELLOWSHIP STIPEND UP TO $40,000**
- **INSTITUTIONAL GRANT UP TO $15,000**

Applicants must be:

- **African American**
- **Appointed as a postdoctoral fellow during the 1996 calendar year at an academic institution or a non-academic institution, such as the National Institutes of Health or the Centers for Disease Control**
- **Committed to conduct research at a Merck research facility for a minimum of three months**
- **A U.S. citizen or permanent resident.**

For application packets, contact:
Rebecca Bennett, The College Fund/UNCF, 1-800-331-2244

Deadline for receipt is February 26, 1996

"A mind is a terrible thing to waste."

Executive Director

American Association for Accreditation of Laboratory Animal Care (AAALAC)

The Executive Director is the Chief Executive Officer of the American Association for Accreditation of Laboratory Animal Care in Rockville MD to start July 1996. Duties are in four areas: **1) operation of the AAALAC office; 2) fiscal and personnel management for the Association; 3) oversight of the accreditation program; 4) professional spokesperson for the organization**. In all matters, the Executive Director accepts policies formulated by the Board of Trustees and the Executive Committee and implements programs consistent with those policies.

The following qualifications are required: An earned post-graduate degree; Familiarity with the function of NIH, USDA, and other federal agencies in animal care and use programs; Ability to communicate the position of AAALAC to professional and public constituencies. The following qualifications are desirable: Expertise in the management of non-profit organizations; Experience in the administration of animal care and use programs; Familiarity with the role of AAALAC as an accrediting agency, and with the issues of animal care and use in research, teaching, and testing.

Send Resume and at least 3 letters of recommendation to: **Hugh L. Evans, Ph.D., Chair, AAALAC Search Committee, Nelson Institute of Environmental Medicine, New York University Medical Center, Long Meadow Rd., Tuxedo, N.Y. 10987.**

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mayo

MAYO CLINIC
MAYO CANCER CENTER

Associate Director for Cancer Prevention and Control

The NCI-designated Mayo Cancer Center in Rochester, Minnesota, invites applications for Associate Director of Cancer Prevention and Control. Investigators with established research programs in cancer prevention and control and with demonstrated leadership skills are encouraged to apply. The Mayo Clinic provides an outstanding environment for conducting cancer prevention and control research, with particular strengths in epidemiology biostatistics and health services research. Interested candidates should submit a letter of application including a statement of research interests, curriculum vitae, bibliography, and list of references to:

James Ingle, M.D.
Associate Director for Clinical Research
Mayo Cancer Center
Mayo Clinic
200 First Street SW
Rochester, MN 55905

Mayo Foundation is an affirmative action and equal opportunity educator and employer.

POSITIONS OPEN

SCIENCE EDUCATOR
PHYSICAL SCIENTIST

The College of Science seeks an **ASSISTANT** or **ASSOCIATE PROFESSOR**, tenure track, to begin August 1996. A doctorate is required in atmospheric sciences, geology, or physics coupled with knowledge and experience in pre-college teaching and teacher preparation. Teaching will be at least 50% in the Science Education Program, including Secondary Science Methods and Student Teacher Supervision. Appointment and science teaching will be in the appropriate science department. Scholarship in physical science and/or teaching and learning, including research and publication and external funding, is expected. San Jose State University is a multicultural metropolitan California State University in the Silicon Valley, with a wide array of academic programs for its diverse population of about 26,000 students.

Curriculum vitae, statement of academic priorities, and three letters of reference must be received by February 16, 1996. Send to: **Dr. Jean Beard, College of Science, San Jose State University, San Jose, CA 95192-0099.** *San Jose State University is an Equal Opportunity/Affirmative Action Employer.*

COGNITIVE SCIENCE

The University of Alabama at Birmingham (UAB) invites applications and nominations for a position in Cognitive Science. Candidates must have a strong record of research accomplishment and are expected to maintain an active extramurally funded research program. The successful candidate is expected to take a leadership role in a new interdepartmental graduate program in Cognitive Science. The appointment will likely be at the **ASSOCIATE** or **FULL PROFESSOR** level but applications from excellent candidates at the Assistant Professor level are also welcome. Review of applications will begin immediately and continue until the position is filled. Applicants should submit a curriculum vitae, summary of research interests, representative reprints, and the names of three referees to: **Michael Sloane, Ph.D., Cognitive Science Search Committee, Department of Psychology, UAB, Birmingham, AL 35294-1170. FAX: 205-975-6110; Email: sloane@eyes.uab.edu.** *UAB is an Equal Opportunity/Affirmative Action Employer.*

MALACOLOGIST
AMERICAN MUSEUM OF NATURAL HISTORY

The Department of Invertebrates invites applications for a **TENURE-TRACK** curatorial position in malacology to be available September 1, 1996. The prime requisite is a strong program in collections-based research in malacology including active fieldwork and appropriate laboratory and analytic approaches. The focus should be in one or more of the following areas: systematics, phylogenetic analysis, biogeography, analyses of life history or other ecological traits, or evolution. The new curator will also be responsible for managing the Department's large collection of molluscs in addition to participating in Museum committees, educational programs, and exhibition programs. The salary will be commensurate with qualifications and experience. The successful candidate must have the Ph.D. by time of appointment. Applications should include (1) a brief statement of research interest; (2) curriculum vitae; (3) complete bibliography; and (4) three letters of recommendation from persons familiar with the applicant's qualifications. Applications must be submitted by February 15, 1996 to: **Dr. Neil H. Landman, Chairman, Search Committee, Department of Invertebrates, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024.** *An Equal Opportunity Employer.*

CARNEGIE MELLON UNIVERSITY

Carnegie Mellon's Department of Engineering and Public Policy has **FACULTY OPENINGS** for people with Ph.D.'s in engineering or science to teach and do research on: the social dimensions of global change; manufacturing and industrial policy; arms control and security policy. Résumé with publication list and references to: **Granger Morgan, Head EPP, Carnegie Mellon, Pittsburgh, PA 15213.**

POSITIONS OPEN

ACADEMY OF NATURAL SCIENCES
Director—Environmental Research Center

The Academy of Natural Sciences has an immediate opening for **DIRECTOR** of its Patrick Center for Environmental Research, in Philadelphia. The Center's staff of over 40 full-time professionals (including 10 Ph.D.s) perform research on the effects of natural processes and human activities on the structure and function of aquatic ecosystems, and apply this knowledge to the sound management of watersheds. The Director is responsible for all aspects of the Center's operation, including program, fiscal, and general management, and coordination with the Academy's other research centers. Requires Ph.D. in an appropriate environmental/ecological discipline, a strong understanding of and commitment to basic and applied ecological research, and demonstrated leadership experience in an academic research setting. The successful candidate will be articulate, highly organized, and capable of using excellent interpersonal skills to interact well with Patrick Center scientists, the Academy's administration, potential sponsors in governmental agencies and the private sector, and the public. The Director must develop and lead an aggressive marketing effort aimed at obtaining grants and contracts from governments and industries concerned with the environmental quality of aquatic ecosystems. Send curriculum vitae and three letters of reference by January 15, 1996 to: **Chair, PCER Director Search Committee, Academy of Natural Sciences, 1900 Benjamin Franklin Parkway, Philadelphia, PA 19103-1195.** Full benefit package. *Affirmative Action/Equal Opportunity Employer.*

The Denver Museum seeks applicants for **CHAIR** and **CURATOR** in the Department of Earth Sciences. The Chair will supervise a department of three curators, a collection manager, office manager, two preparators, and over 100 volunteers. Collection strengths are in fossil vertebrates and plants, and minerals from Western North America (about 30,000 specimens each). The successful candidate must be committed to museum exhibition, education, and research, and be able to manage and teach in a certification program in paleontology for amateurs with an emphasis on terrestrial ecosystems. Experience in managing large projects and budgets and a record of successful grant-funded research is required. Candidates must have a Ph.D. with demonstrated research excellence in area of collection strengths of the Museum and at least five years' experience as a curator. Applicants should submit a letter of application, curriculum vitae, three publications (scientific and popular), and arrange to have three letters of recommendation sent to: **Personnel Office, Denver Museum of Natural History, 2001 Colorado Boulevard, Denver, CO 80205.** Closing date is January 9, 1996. **FAX: 303-331-6492; Email: paleo@dmnh.org.** *The Denver Museum is an Affirmative Action/Equal Employment Opportunity/ADA Employer.*

ASSISTANT PROFESSOR
HUMAN ANATOMY

The Department of Anatomy, Northeastern Ohio Universities College of Medicine, is seeking a **TENURE-TRACK FACULTY MEMBER** to participate in teaching medical gross anatomy/embryology, and to contribute to the research program of the Department. Applicants should have an active research program in morphological aspects of mammalian evolution and experience in teaching dissection-based human gross anatomy. The Department is interested in expanding its strengths in functional morphology, paleontology, and/or biological anthropology.

The Northeastern Ohio Universities College of Medicine is part of a consortium that includes Kent State University, The University of Akron, and Youngstown State University. Also included in the research base of the consortium is the Cleveland Museum of Natural History. A doctoral program in biological anthropology is offered through the School of Biomedical Sciences at Kent State University. Applications consisting of a cover letter stating research interests, curriculum vitae, and three letters of recommendation should be sent to: **Dr. Steve Ward, Department of Anatomy, Northeastern Ohio Universities College of Medicine, P. O. Box 95, 4209 State Route 44, Rootstown, OH 44272.** The appointment begins July 1, 1996. The deadline for applications is February 15, 1996. *Affirmative Action/Equal Opportunity Employer.*

POSITIONS OPEN

FACULTY POSITIONS
The University of Texas
Southwestern Medical Center
Hamon Center for Basic Research in Cancer

The Hamon Center for Basic Research in Cancer at The University of Texas Southwestern Medical Center invites applications for tenure-track faculty positions at the level of **ASSISTANT** or **ASSOCIATE PROFESSOR**. We are seeking individuals who will interact effectively within a center with strengths in developmental biology, molecular biology, and genetics. Successful applicants should be capable of establishing a vigorous independent research program and should be prepared to participate in teaching in one of several vigorous graduate programs. Scientists working in the areas of signal transduction, gene regulation, and differentiation are particularly encouraged to apply.

Applicants should submit a curriculum vitae containing a brief summary of past research accomplishments, a statement of future research objectives, and three letters of reference to:

Dr. Eric N. Olson, Director
Hamon Center for Basic Research in Cancer
University of Texas
Southwestern Medical Center at Dallas
5323 Harry Hines Boulevard
Dallas, TX 75235-9038

An Equal Opportunity Employer.

DEVELOPMENTAL BIOLOGIST: The Department of Biology, Hofstra University, invites applications for an **ASSISTANT PROFESSOR** tenure-track position. Applicant must be capable of maintaining an active research program involving undergraduate and Master's degree students and developing a modern laboratory/lecture course in developmental biology. Teaching experience in embryology desired. Ph.D. required; postdoctoral experience preferred. Submit curriculum vitae, statements of teaching goals and research interests (including a short description of plan to involve students in research), three representative reprints, and three letters of reference to: **Developmental Biologist Search, Dr. Dorothy E. Pumo, Chair, Department of Biology, 114s Hofstra University, Hempstead, NY 11550.** Deadline: 31 January 1996. *Hofstra University is an Equal Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.*

RENSSELAER POLYTECHNIC INSTITUTE, Department of Chemistry invites applications for a tenure-track **ASSISTANT PROFESSORSHIP** in Analytical/Biochemistry to begin fall 1996. Particular consideration will be given to applicants with research interests in biological, biomaterials or environmental applications of analytical chemistry. Exceptional candidates may be considered for appointment at a higher rank. Applications should include a curriculum vitae with a brief description of proposed research projects and letters of recommendation from three professional references. Applications must be submitted by January 30, 1996 and sent to: **Professor Joseph T. Warden, Chair, Faculty Search Committee, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180-3590.** *Rensselaer Polytechnic Institute is an Equal Opportunity/Affirmative Action Employer and encourages applications from women and minority candidates.*

GENETICIST/DEVELOPMENTAL BIOLOGIST

The Whittier College Biology Department seeks **ASSISTANT PROFESSOR** for tenure-track position, subject to funding (to begin fall term, 1996), to teach lectures and labs for upper-division courses in genetics and developmental biology. Duties will also include teaching introductory biology for majors and non-majors. Faculty are expected to establish a research program that involves undergraduates. Ph.D. required; postdoctoral experience preferred. Submit letter of application (including statements of teaching and research interests), curriculum vitae, and three letters of reference that evaluate teaching and research. Files will be reviewed beginning January 31, 1996, continuing until position is filled. Send all documents to: **Dr. Clifton Morris (Chair), Biology Department, Whittier College, P.O. Box 634, Whittier, CA 90608.** *Equal Opportunity/Affirmative Action Educator/Employer.*

UMD NEW JERSEY

NEW JERSEY MEDICAL SCHOOL

CHAIRPERSON

Department of Pharmacology and Physiology

The New Jersey Medical School of the University of Medicine and Dentistry of New Jersey invites nominations and applications for the position of Chairperson of the Departments of Pharmacology and Physiology. We intend to propose the merger of these two departments in the near future. The successful candidate will have the opportunity to mold and develop the educational and research programs of the new department created by this merger. The individual selected as chair must have a nationally recognized record of achievement in research and a strong interest in the development of two major teaching programs in the Medical School and the Graduate School.

For further information or to apply, please send your curriculum vitae to:

Ms. Linda C. Reich
NJMS - Office of the Dean
UMDNJ-New Jersey Medical School
University Heights
185 South Orange Ave., MSB - C662
Newark, NJ 07103-2714

The UMDNJ is an Affirmative Action/ Equal Employment Opportunity Employer M/F/D/V and is a member of the University Health System of NJ.

TOXICOLOGIST

Rohm and Haas Company, a leading chemical company serving global markets, has an immediate opening for a Toxicologist with expertise in *in vitro* methodology. This position is at our Corporate Research Center in Spring House, PA, near Philadelphia.

We are looking for a scientist to assume the leadership of a laboratory conducting assays in genetic toxicology, as well as research in toxicology using *in vitro* methods. The assays and research are primarily directed toward the expeditious development of new products for regulatory acceptance.

The successful candidate will have a Ph.D. degree in a relevant discipline, with demonstrated technical expertise in *in vitro* toxicology methods: e.g., genetic toxicology, cytotoxicity, receptor-mediated toxicity. This scientist will also have a substantive publication record in the peer-reviewed literature.

Our position requires a broad perspective of toxicology, as well as outstanding oral and written communication skills to interact effectively with regulatory scientists and our company's scientific staff. Candidates must be skilled in project management and have the drive, aptitude and motivation to work effectively in multidisciplinary teams.

Rohm and Haas offers a highly competitive salary and benefits program, including relocation assistance. We invest heavily in research and in resources to develop our Research staff. Candidates must be legally qualified to hold regular employment in the U.S. For consideration, please forward your resume or C.V., including publication list and references, to: **Corporate Staffing #295, Rohm and Haas Research Laboratory B-70, 727 Norristown Road, Spring House, PA 19477-0904.**

ROHM AND HAAS

An Equal Opportunity Employer M/F/H.

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

USDA National Needs Graduate Fellowships

Here's your opportunity to be part of one of the nation's leading plant biotechnology programs. Iowa State is offering graduate fellowships in cell biology, biochemistry, molecular biology and physiology through the USDA National Needs Graduate Fellowship program. Fellowships are in Iowa State's Interdepartmental Plant Physiology Major (IPPM).

Fellowships are for three years with an annual stipend of $17,000. Candidates with a master's degree or prior research experience are preferred.

For more information and application details about these fellowships and others, contact: IPPM Graduate Admissions, 353 Bessey Hall, Iowa State University, Ames, Iowa 50011; email: ippm@iastate.edu OR http://www.public.iastate.edu/~ippm; phone 515-294-0132; fax 515-294-1337.

Iowa State University is an affirmative action/ equal opportunity employer.

Cubist Pharmaceuticals, Inc.

KEEP PACE WITH DISCOVERY

Cubist Pharmaceuticals is racing toward critical medical discoveries. We're dedicated to the development and commercialization of fundamentally new approaches to anti-infective drugs. Your insight and expertise can help us make this goal a reality.

Enzymology

Positions currently exist for scientists with expertise in enzymology. Successful candidates will have a Ph.D. in Biochemistry, previous industrial experience, and a proven track record in enzymology with relevant publications. Candidate must be able to function in the small biotech environment with emphasis on independence, creativity and excellent science. Responsibilities include development and execution of strategy to achieve scientific objectives, protein purification, assay development, kinetic and mechanism of action studies.

Protein Chemistry

Position currently exists for scientist with expertise in protein chemistry. Successful candidates will have a Ph.D. in Biochemistry or equivalent, a minimum of 2 years' experience in protein purification using HPLC and ligand-affinity techniques, and experience in supplying proteins for X-ray crystallography or NMR studies. Responsibilities include taking the lead in expressing and purifying enzymes and proteins that are targets for drug discovery programs; working with molecular biologists and biochemists in target selection and optimization of enzymatic assays; and playing a central role in developing and supporting structural biology programs involving X-ray crystallography, NMR and protein mapping.

We're setting an intense pace for research and discovery, balanced by exciting professional opportunities. Working with us, you'll enjoy a competitive compensation package. Please send your resume to: **Manager, Human Resources, Cubist Pharmaceuticals, Inc., 24 Emily Street, Cambridge, MA 02139.** We're an equal opportunity employer.

PROFESSOR AND CHAIRPERSON DEPARTMENT OF BIOLOGY

Northeastern University seeks applications and nominations for a **CHAIRPERSON** for the Department of Biology. The department consists of 22 full-time faculty, 28 professional staff and postdoctoral fellows, 114 graduate students, and 300 undergraduate majors. Research areas with significant extramural grant support include biochemistry, cell and molecular biology, physiology, marine biology, and ecology. The department participates in interdisciplinary programs in biochemistry, neuroscience, and marine biology. Northeastern University, a Carnegie II Research Institution, benefits from its Boston location and the close proximity of our Marine Science Center.

The department seeks an innovative and dynamic individual to fill the position by September 1996. Candidates must have a Ph.D., an internationally recognized record of scholarly research, proven administrative and leadership skills, and must qualify for appointment as full professor with tenure. The chairperson will be expected to maintain a well-funded research program, to provide outstanding leadership in enhancing both research and educational programs at the undergraduate and graduate levels, and to have a demonstrated commitment to issues of diversity.

Applicants should submit their curriculum vitae, a letter describing their qualifications for the position, and three letters of recommendation to: **Carol M. Warner, Biology Search Committee Chair, Faculty Senate Office, 442 Ryder, Northeastern University, Boston, MA 02115.** Full consideration will be given to applications received by February 15, 1996. *Northeastern University is an Equal Opportunity/Affirmative Action/Title IX Employer.*

Aquatic Invertebrate Biologist, **ASSISTANT PROFESSOR.** The Department of Biology and Microbiology, University of Wisconsin-La Crosse invites applications for an academic year, tenure-track position at the Assistant Professor level to start August 26, 1996. Applicants should have a strong commitment to undergraduate education. The successful candidate must have a Ph.D. and will be expected to (1) teach a course in aquatic invertebrate biology, (2) develop a course in an area of specialization, (3) assist in lectures and laboratories in introductory biology and/or zoology, and (4) develop an externally funded research program and direct undergraduate and graduate (M.S.) research. Candidates must have background and/or experience in one or more of the following areas: ecotoxicology, molecular toxicology, transport and fate of contaminants, environmental impact and risk assessment, GIS, physiological ecology. Send curriculum vitae, statements of teaching philosophy and research interests, graduate and undergraduate transcripts, and three letters of recommendation to: **Dr. James E. Parry, Chairman, Department of Biology and Microbiology, University of Wisconsin-La Crosse, La Crosse, WI 54601.** Deadline for receipt of materials is February 15, 1996. *The University of Wisconsin-La Crosse is an Affirmative Action/Equal Opportunity Employer. Women and minorities are encouraged to apply.*

Geneticist, **ASSISTANT PROFESSOR.** The Department of Biology and Microbiology, University of Wisconsin-La Crosse invites applications for an academic-year tenure-track Assistant Professor, to begin August 26, 1996. Applicants should have a strong commitment to undergraduate education and must have a Ph.D. in genetics with an emphasis in plant molecular genetics. The successful candidate will be expected (1) to teach a general genetics course, (2) to develop a course in an area of specialty, (3) to participate in a plant biology course and (4) to develop an externally funded research program and to direct undergraduate and graduate (M.S.) research. Send curriculum vitae, statements of teaching philosophy and research interests, graduate and undergraduate transcripts and three letters of recommendation to: **Dr. James E. Parry, Chairman, Department of Biology and Microbiology, University of Wisconsin-La Crosse, La Crosse, WI 54601.** Deadline for receipt of materials is February 23, 1996. *The University of Wisconsin-La Crosse is an Affirmative Action/Equal Opportunity Employer. Women and minorities are encouraged to apply.*

POSTDOCTORAL POSITION available immediately to carry out structure/function analysis of a novel class of small Zn^{2+}-binding transcriptional activators. Applicants should have a strong background in protein biochemistry, including experience with protein overexpression and purification. Send curriculum vitae, statement of research interests, and names of three references to: **Dr. Gail Christie, Department of Microbiology and Immunology, Virginia Commonwealth University, Box 980678, Richmond, VA 23298-0678.**

NORTH CAROLINA CENTRAL UNIVERSITY DIRECTOR
Biomedical/Biotechnology Research Institute

The **DIRECTOR** of the Biomedical/Biotechnology Research Institute will provide leadership for a broadly based modern research and training program that includes a variety of subdisciplines, such as environmental health, toxicology, and carcinogenesis. Responsibilities include setting research priorities; recruiting and hiring a staff of investigators and guiding them to secure external research and training funding; and insuring collaborative interactions among other relevant North Carolina Central University (NCCU) programs, local universities, and federal and corporate research communities.

Candidates for the position must hold the Ph.D. or equivalent doctoral degree, preferably in the biomedical sciences, have expertise in one of the biomedical research disciplines, and be an experienced scientist, academician, and administrator. The applicant should have published in refereed journals, possess an extensive record of research funding, and be experienced in directing an established and successful research program.

This is a twelve-month appointment. Rank and salary depend upon qualifications and experience. Review of candidates will begin on February 19, 1996, and will continue until the position is filled.

Please send a letter of application, curriculum vitae, and names of references to: **The BBRI Search Committee, c/o Gloria A. Haynes, Personal Assistant to the Chancellor, North Carolina Central University, Post Office Box 19617, Durham, NC 27707.** *NCCU complies with the Immigration Reform and Control Act of 1986.*

ASSOCIATE PROFESSOR CANCER PHARMACOLOGY

The School of Pharmacy and the Norris Cancer Center, University of Southern California (USC), Los Angeles, are seeking experienced investigators with background in chemistry, biochemistry, or molecular biology for a **TENURED/TENURE-TRACK** position. Persons conducting research in the general area of cancer pharmacology, and specifically oriented toward novel therapies based on molecular mechanisms, will be given the highest consideration. Teaching at the graduate and professional levels in the School of Pharmacy is required. Candidates should send the names of three individuals who can provide letters of reference and should send a curriculum vitae and a brief summary of research accomplishments and future research goals to: **Michael B. Bolger, Ph.D., School of Pharmacy, University of Southern California, 1985 Zonal Avenue, PSC 700, Los Angeles, CA 90033.** Evaluation of applications will begin February 1, 1996 and continue until the position is filled. *USC is an Equal Opportunity/Affirmative Action Employer.*

Mycologist, **ASSISTANT PROFESSOR.** The Department of Biology and Microbiology, University of Wisconsin-La Crosse invites applications for an academic year, tenure-track position at the Assistant Professor level to start August 26, 1996. Applicants should have a strong commitment to undergraduate education. The successful candidate will be expected to teach general mycology and medical mycology, and to participate in introductory microbiology and/or biology courses. The successful candidate will be expected to develop an externally funded research program and direct undergraduate and graduate (M.S.) research. A Ph.D. required with an emphasis in mycology. Send curriculum vitae, statment of teaching philosophy and research interests, graduate and undergraduate transcripts, and three letters of recommendation to: **Dr. James E. Parry, Chair, Department of Biology and Microbiology, University of Wisconsin-La Crosse, La Crosse, WI 54601.** Deadline: materials to be received by February 15, 1996. *The University of Wisconsin-La Crosse is an Affirmative Action/Equal Opportunity Employer. Women and minorities are encouraged to apply.*

POSTDOCTORAL POSITION available to study the mechanisms and genetics of human retinal degenerations. Experience with genetic screening, cloning, expression and site-directed mutagenesis preferable. Funding is available for two years with the potential for evolving into an independent self-supporting investigator in an active research environment. Send curriculum vitae and names of three references to: **Ted Acott, Ph.D., CERES, Casey Eye Institute, Oregon Health Sciences University, 3375 SW Terwilliger, Portland, OR 97201. Email: acott@ohsu.edu.** *Equal Opportunity Institution.*

FACULTY POSITION IN X-RAY CRYSTALLOGRAPHY

The Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School and the Dana-Farber Cancer Institute are seeking applications for a position at the **ASSISTANT PROFESSOR** level to be located at the Dana-Farber Cancer Institute. Applicants should have an interest in the application of x-ray crystallography to address significant problems in structural biology, biochemistry, pharmacology, and cancer. The successful candidate will join an outstanding interactive group of structural biologists in the Harvard Medical School community. Interested applicants should send an outline of future research plans, a curriculum vitae, bibliography, and the names of three to four references to: **Professor James M. Hogle, Search Committee, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115.** *Women and minorities are encouraged to apply. Harvard University and Dana-Farber Cancer Institute are Equal Opportunity Employers.*

POSTDOCTORAL APPOINTMENT available for study of the role of oxidative stress in Alzheimer's disease and aging. Experience with contemporary molecular biology and protein biochemistry methods is desired. Our research emphasizes recombinant protein processing and trafficking. The Department of Pathology at the University of Texas provides stimulating scientific atmosphere and is part of the Texas Medical Center, the world's largest medical and scientific conglomerate. This also provides unlimited possibilities for collaborative research. Candidates should send curriculum vitae, transcripts, statement of research interests, and three letters of reference to: **Miguel Pappolla, M.D., Department of Pathology and Laboratory Medicine, University of Texas at Houston, P. O. Box 20708, Houston, TX 77225. Email: pappolla@casper.med.uth.tmc.edu.** *The University of Texas is an Equal Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.*

UNIVERSITY OF CINCINNATI POSTDOCTORAL POSITIONS
Department of Pathology and Laboratory Medicine

The Department of Pathology and Laboratory Medicine at the University of Cincinnati is recruiting for two **POSTDOCTORAL POSITIONS** for those scientists with a recent doctorate degree and a strong background in molecular biology techniques. The successful candidate's studies will involve the generation and use of transgenic and gene knockout mice in delineating the mechanisms for dietary regulation of lipid absorption genes and in the relationship between dietary lipid absorption and atherosclerosis.

Interested applicants should send a curriculum vitae and the names of three references. Applications will be accepted until the position is filled. Applications and inquiries should be directed to:

David Hui, Ph.D.
Department of Pathology and Laboratory Medicine
University of Cincinnati College of Medicine
P.O. Box 670529
Cincinnati, OH 45267-0529

Vertebrate Zoology, Biology Department, College of Staten Island, City University of New York (CUNY), anticipates a tenure-track appointment as **ASSISTANT PROFESSOR** for September 1996. Ph.D., college instruction, curriculum development, and record sufficient for appointment to CUNY graduate faculty required. Postdoctoral experience and grant activity preferred. Send curriculum vitae, research plans, and names and addresses of three references to: **Prof. J. LeBlanc, Search Committee Chair, Biology Department, College of Staten Island/CUNY, 2800 Victory Boulevard, Staten Island, NY 10314.** *Equal Employment Opportunity/Affirmative Action/ADA Employer.*

POSTDOCTORAL FELLOWSHIPS
Molecular and Cell Biology of Apolipoproteins

Positions are available to study the regulation and functions of apolipoproteins involved in lipid transport in both cell and transgenic mouse models. Candidates must have a Ph.D., M.D., or both. A strong background in cell and/or molecular biology is preferred. Positions available through an NIH training grant: *U.S. citizens or permanent residents only.* Contact: **Ms. Irene Franck, Division Administrator, Department of Medicine, P&S 9-510, Columbia University, 630 West 168th Street, New York, NY 10032.** *We take Affirmative Action toward Equal Opportunity.*

The DEPARTMENT OF BIOCHEMISTRY at the University of Alberta invites applications for a FACULTY POSITION preferably at the Assistant Professor level (Salary Range $39,230-$55,526). This position is an entry into the regular academic progression of the university. The candidate is expected to secure extramural funding to support a high quality research program in an area that complements existing strengths of the department. These include: control of protein and mRNA turnover, molecular biology of lipids and lipoproteins, molecular immunology and virology, protein structure and function, proteolysis mechanisms, recombination and repair, signal transduction and transcriptional control (see http://www.biochem.ualberta.ca). We offer an outstanding research environment and graduate training program in these areas. An attractive start-up package, including three years of initial salary support, will be provided. In accordance with Canadian Immigration regulations, priority will be given to Canadian citizens and permanent residents of Canada. Individuals with a Ph.D. or equivalent and appropriate postdoctoral experience should send a letter of application, curriculum vitae, brief description of research plans, and arrange to have three letters of reference sent to: **Dr. Vern Paetkau, Chair, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.** The application deadline is January 31, 1996. The University of Alberta is committed to the principle of equity in employment. As an employer we welcome diversity in the workplace and encourage applications from all qualified women and men, including Aboriginal peoples, persons with disabilities and members of visible minorities.

RESEARCH SCIENTIST
CELLULAR/ MOLECULAR IMMUNOLOGIST

Millennium Pharmaceuticals Inc. has fantastic opportunities in their Biology Division for a team of enthusiastic and creative cellular and molecular biologists and immunologists. Join a group focused on the identification and characterization of the biological function of novel genes as therapeutic targets for the treatment of inflammatory respiratory diseases including asthma, chronic bronchitis and COPD. The following positions are currently available:

RESEARCH SCIENTIST

The successful candidate will have demonstrated outstanding ability with a biological focus in the areas of lymphocyte activation and effector function. A Ph.D. in Molecular Biology or a related field and strong skills in cellular and molecular immunology are required.

RESEARCH ASSOCIATES

Positions will include the functional characterization of novel proteins expressed in cells of the immune system. Candidates will have a variety of responsibilities including mammalian cell culture, molecular biology, protein purification and analysis, and cellular biology. An MS and 3-5 years of experience is required.

Millennium is a rapidly growing biopharmaceutical company researching the genetic basis of common diseases and developing therapeutics using this understanding. Enjoy the rewards of an innovative environment, the challenge of progressive science and the incentive of stock ownership and a generous compensation package. Publications in leading scientific journals actively encouraged. Please send or fax your C.V. to: **Dr. Douglas Levinson c/o Shani Stickney, Millennium Pharmaceuticals, Inc., 640 Memorial Drive, Cambridge, MA 02139; FAX (617) 225-0884.** EOE.

MILLENNIUM
PHARMACEUTICALS
Pathways To Discovery

TEXAS A&M UNIVERSITY

Postdoctoral Positions and Graduate Assistantships in Plant Virology and Plant Biotechnology

Available immediately in the following research programs of the respective principal investigators.

1. Molecular characterization of furoviruses
   Drs. Charlie Rush and Karen-Beth Scholthof
2. Satellite Viruses and Satellite RNAs
   Drs. Karen-Beth Scholthof and Marilyn Roossinck
3. Long Distance Movement of Tombusviruses
   Dr. Herman B. Scholthof
4. Interactions Between Different Furoviruses
   Dr. Charlie Rush
5. Engineered Resistance and Molecular Farming in Transgenic Sugarcane and Sorghum
   Drs. T. Erik Mirkov and James Irvine

For category 1-3, send applications or requests for additional information to the **Department of Plant Pathology and Microbiology at Texas A&M University, College Station, TX 77843**, Fax: 409-845-6483. For category 4, send correspondence to the **Texas Agricultural Experiment Station (TAES), P.O. Drawer 10, Bushland, TX 79012**, Fax: 806-354-5829, and for category 5 to **TAES, 2415 E Hwy 83 Weslaco, TX 78596**, Fax: 210-968-0641, or e-mail:e-mirkov@tamu.edu.

mayo

MAYO CLINIC
MAYO CANCER CENTER

Staff Scientist in Cancer Research

The NCI-designated Mayo Cancer Center in Rochester, Minnesota, invites applications for several staff scientist positions in cancer research. Investigators with established research programs in areas of cancer biology with clinical/translational applicability are especially encouraged to apply. Areas of particular emphasis in the Mayo Cancer Center include breast and gynecologic malignancies, neuro-oncology (adult and pediatric primary brain tumors), prostate and genito-urinary malignancies, gastrointestinal malignancies, soft tissue sarcomas (including pediatric solid tumors), and hematologic malignancies. The Mayo Clinic provides an outstanding environment for investigators interested in applying modern advances in the cellular and molecular basis of cancer to improved methods in the prevention, diagnosis and treatment of cancer. Interested applicants should submit a statement of research interests, curriculum vitae, bibliography and list of references to:

Nita J. Maihle, Ph.D.
Associate Director for Basic Research
Mayo Cancer Center
Mayo Clinic
200 First Street SW
Rochester, MN 55905

Mayo Foundation is an affirmative action and equal opportunity educator and employer.

POSITIONS OPEN

POSTDOCTORAL POSITION EPITHELIAL STEM CELLS

Position available to study the biological properties of corneal and conjunctival epithelial stem cells. Candidates must have a Ph.D. and/or M.D. with a strong background in cell culture and molecular biology. Additional experience in conducting studies with laboratory animals (rodents, rabbits) is a strong asset. Send curriculum vitae, including description of research experience and letters of three references to: **Robert M. Lavker, Ph.D., Department of Dermatology, University of Pennsylvania Health System, 235 CRB, Philadelphia, PA 19104-6142.**

University of Pennsylvania is an Affirmative Action/Equal Opportunity Employer.

POSTDOCTORAL FELLOWSHIP

A **POSTDOCTORAL FELLOWSHIP** is available in protein chemistry. Incumbent will study the structural changes of proteins and starch-protein interactions during food processing. Salary is $30,000.00 per year. Applicants with doctoral degree and a strong background in protein chemistry and/or starch-protein interaction should apply to: **Dr. Gour Choudhury, Associate Professor, Fishery Industrial Technology Center, University of Alaska, 900 Trident Way, Kodiak, AK 99615, USA. Telephone: 907-486-1535.**

POSTDOCTORAL FELLOWS wanted for electrophysiological and electrochemical studies of transmitter release using midbrain dopamine and PC12 cell lines. Individuals will collaborate with group providing transfected lines expressing mutant synaptic proteins. Applicants must have Ph.D. or M.D. with equivalent experience. Candidates should have electrophysiology experience. Strong interest in synaptic plasticity desirable. Send curriculum vitae and names of references to: **Dr. David Sulzer, Department of Neurology, Parkinson's Disease Research Center, Black Building 3-307, 630 West 168th Street, New York, NY 10032.** *Columbia University is an Equal Opportunity/Affirmative Action Employer.*

RESEARCH SCIENTIST CELL ANALYSIS

AmCell Corp. is looking for an innovative Ph.D. level scientist to develop and optimize assays for multi-parameter cell analysis. The position requires expertise in bioorganic chemistry and physical chemistry relating to the synthesis of fluorescent labels, their conjugation to biomolecules, and hands-on experience with immunofluorescence, FISH techniques, flow cytometry, and/or fluorescence microscopy. The candidate must have three years of related experience in a research environment and must have excellent written and oral communication skills. Interested candidates please send applications to: **AmCell Corp., Attention: Diether Recktenwald, Ph.D., VP R&D, 1190 Bordeaux Drive, Sunnyvale, CA 94089. Telephone: 408-752-1200; FAX: 408-752-1212.**

POSTDOCTORAL POSITION

University of California and Veterans Administration Medical Center (VAMC), San Francisco, has a position available immediately for a qualified individual to join a group studying the regulation of breast cancer by growth factors and hormones including vitamin D. Applicants should have a Ph.D. in molecular and/or cellular biology. Please send curriculum vitae and names of three references to: **Daniel Bikle, M.D., Ph.D., Department of Medicine, VAMC (111N), 4150 Clement Street, San Francisco, CA 94121.**

POSTDOCTORAL RESEARCH ASSOCIATES Molecular Oncology

Positions available immediately in a laboratory developing new methods for diagnosis and therapy of breast and gynecological cancer. The candidate must be less than three years after obtaining a Ph.D. in biochemistry or molecular biology, and have documented experience with PCR, DNA, and RNA analysis and gene cloning. Candidates will be involved in cloning and characterization of the new female cancer virus and PCR analysis of human samples.

Please send or fax curricula vitae and names of three references to: **Dr. Eva M. Rakowicz-Szulczynska, Associate Professor, Director, Molecular Oncology Laboratories, Department of Obstetrics and Gynecology, University of Nebraska Eppley Cancer Center, 600 South 42nd Street, Omaha, NE 68198-3255. FAX: 402-559-8112.**

FELLOWSHIPS IN MATHEMATICS AND MOLECULAR BIOLOGY

The Program in Mathematics and Molecular Biology has GRADUATE and POSTDOCTORAL FELLOWSHIP support available. Current topics in the Program include geometry, topology, and sequence analysis of DNA, molecular dynamics, phylogenetic comparison, and RNA, and algorithms for molecular structure prediction. Other areas will be considered. Fellowships can be held at any university or college in the United States. Postmark deadline for applications: February 1, 1996. *Women and minorities are encouraged to apply.* Apply to: **PMMB, 103 Donner Lab, Number 3206, University of California at Berkeley, Berkeley, CA 94720-3206. Email: sylviaj@violet.berkeley.edu.**

POSTDOCTORAL POSITION

A **POSTDOCTORAL POSITION** is available to study structural/functional aspects of LDL receptor family members. Projects include investigating the role of LRP in catabolism of amyloid precursor protein (see *Cell*, 82:331–340, 1995), and the role of the VLDL receptor in regulating proteinase catabolism in vascular cells (see *J. Biol. Chem.*, 270:26550–26557, 1995). Submit curriculum vitae with the names and addresses of three references to: **Dr. Dudley K. Strickland, Holland Laboratory, Department of Biochemistry, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855.** *Equal Opportunity Employer.*

POSTDOCTORAL POSITION in molecular biology of nuclear hormone receptors, primarily studying mechanisms of gene regulation by thyroid hormone, retinoid and vitamin D receptors. Experience in molecular biology is required. Send curriculum vitae and letters of reference to: **Dr. Ron Koenig, University of Michigan Medical Center, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0678. FAX: 313-936-6684.** *Equal Opportunity/Affirmative Action Employer.*

Boston Heart Foundation, Harvard/MIT Division of Health Sciences and Technology offers a **POSTDOCTORAL POSITION**, focussing on the earliest steps in atherogenesis. Ph.D. degree with experience in molecular biology is essential; protein characterization experience is helpful. Send curriculum vitae and names of three references to: **Dr. Robert S. Lees, Boston Heart Foundation, 139 Main Street, Cambridge, MA 02142. FAX: 617-253-3653; Email: rsl@MIT.edu.**

POSTDOCTORAL POSITION available immediately to study the roles of free radicals in neuronal injury and degenerative disorders. Ph.D. in neuroscience, biochemistry, or related area. Experience in microdialysis, HPLC, and apoptosis are helpful. Send curriculum vitae, three references, and reprints to: **Dr. Danxia Liu, Hall Magnet 1143, University of Texas Medical Branch, Galveston, TX 77555. FAX: 409-747-2182.** *Affirmative Action/Equal Opportunity Employer.*

POSTDOCTORAL FELLOW

For the identification of ataxia genes (**Pulst** *et al., Nature genetics*, 1993) and the analysis of familial brain tumors (**Sainz** *et al., Hum. Mol. Genet.*, 1994), we are looking for two scientists with Ph.D. and established background in positional cloning and molecular genetics. Fax curriculum vitae with three references to: **Cedars-Sinai Medical Center, Job 1950372 at 310-967-0130 or Email: Pulst@CSMC.EDU.**

POSTDOCTORAL POSITION available to develop novel somatic cell genetics strategies for the study of signal transduction. Requires Ph.D. in medical genetics and previous experience in hematopoesis, retrovirology, somatic cell genetics, fluorescence activated cell sorting, and molecular biology. Ability to perform human karyotype analysis essential. Send curriculum vitae by January 15, 1996 to: **Professor Brent Cochran, Department of Physiology, Tufts University, 136 Harrison Avenue, Boston, MA 02111.**

Doctoral Program in Molecular and Cellular Biology and Pathobiology

Interdisciplinary program in fundamental molecular and cellular biology and its application to disease. Competitive stipends of $15,000 available. Contact: **Director, MCBP Program, College of Graduate Studies, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425-2501.**

POSTDOCTORAL POSITION in Cellular and Molecular Neurobiology—The Department of Neurobiology has postdoctoral positions available to study various aspects of molecular, cellular, and developmental neurobiology. Some major areas of interest are cell-cell and cell-substratum adhesion molecules, neurite outgrowth, and nerve regeneration. Specific studies include: analysis of overexpression, mutation or deletion of adhesion molecules in animals; analysis of genes such as Hox and Pax that control the expression of adhesion molecules, and their subsequent roles in the formation of morphogenetic pathways during neural development; identification of signal transduction pathways and the gene programs activated by adhesion molecule binding. Applicants should have a strong background in cellular and/or molecular biology. Send curriculum vitae and three letters of reference to: **Dr. Gerald M. Edelman, Chairman, Department of Neurobiology, The Scripps Research Institute, 10666 North Torrey Pines Road, SBR14, La Jolla, CA 92037.** *Affirmative Action/Equal Opportunity Employer.*

POSTDOCTORAL POSITIONS

TWO positions are available immediately to study *Mycobacterium tuberculosis*. Candidates must have laboratory experience in molecular genetics, automated DNA sequence analysis, or IS6100 typing. Two positions are also available to study *Streptococcus pyogenes* pathogenesis. Candidates must have experience in molecular genetics, recombinant protein expression and purification systems, or a broad background in immunology. Send curriculum vitae, including the names and telephone numbers of three references, to: **James M. Musser, M.D., Ph.D., Section of Molecular Pathobiology, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. FAX: 713-798-4595.** *Baylor College of Medicine is an Equal Opportunity/Affirmative Action and Equal Access Employer.*

POSTDOCTORAL POSITION—Tuberculosis drug development. A Ph.D. molecular biologist to clone, express, and "knock-out" genes required for cell wall biosynthesis in mycobacteria. Work would be part of extensive mycobacterial research done at Colorado State on drug development, physiology, genetics, and immunology of mycobacteria. Contact: **Dr. Michael McNeil, Department of Microbiology, Colorado State University, Fort Collins, CO 80523. Email: mmcneil@vines.colostate.edu; Telephone: 970-491-1784; FAX: 970-491-1815.**

POSTDOCTORAL OR RESEARCH ASSOCIATE POSITION available immediately to study the pathobiology of progressive renal disease in animal models and in cell culture. Techniques include Northern and Western analysis, reverse transcription PCR, in situ hybridization, ELISA detection of cytokines, immunohistochemistry, cell and tissue culture. Send curriculum vitae to: **Jonathan R. Diamond, M.D., Division of Nephrology, The Milton S. Hershey Medical Center, P.O. Box 850, Hershey, PA 17033.** *The Pennsylvania State University is an Equal Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.*

POSTDOCTORAL POSITIONS (two) and one **RESEARCH COORDINATOR** position available immediately to study receptors, G-proteins, and ion channels involved in sensory transduction in vertebrate taste cells (*Nature*, 376:80, 1995; *Nature*, 376:85, 1995; *Nature*, 357:563, 1992). Strong background in molecular biology, protein biochemistry, or transgenic technology required. Send curriculum vitae, names and addresses of three references, and a statement of research interests to: **Dr. R. F. Margolskee, Box 1218, Department of Physiology and Biophysics, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574.** *Equal Opportunity/Affirmative Action Employer.*

POSTDOCTORAL POSITIONS available to study either the cis- and trans-acting components of vaccinia virus DNA replication or the role of protein phosphorylation in regulating viral transcription, replication, and morphogenesis. Submit curriculum vitae and three letters of reference to: **Dr. Paula Traktman, Department of Cell Biology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021. Email: ptrakt@mail.med.cornell.edu.** *Equal Employment Opportunity/Affirmative Action/Minorities/Female/Disabled/Veteran.*

SENIOR SCIENTISTS

Avitech Diagnostics, Inc. is pioneering the development of automated, high throughput systems for genetic diagnosis. We are applying proprietary technology based on nucleic acid enzymology to scan unknown mutations in disease-related genes. Avitech collaborates extensively with leading academic laboratories in the fields of cancer and cardiovascular disease. Our scientists work in an interactive, team-oriented environment that rewards initiative.

TECHNOLOGY DEVELOPMENT

We are seeking a Ph.D. biochemist/molecular biologist to direct projects from the discovery phase through proof-of-principle, validating technology for further commercial development. The successful candidate will have a record of innovation in developing new methods, solving experimental problems, and leading collaborative projects. Interactions with external collaborators will require outstanding oral and written communication skills.

ASSAY DEVELOPMENT

This scientist will adapt gene scanning technology to diagnostic formats compatible with existing automated clinical instruments. We are seeking candidates with at least 3 years' experience in diagnostic development and project management, an in-depth knowledge of clinical instrumentation, and a Ph.D. in biochemistry, molecular biology, or immunology.

Avitech Diagnostics offers highly competitive salary, benefits, & stock options. For immediate consideration, send CV to: **Research Director, Avitech Diagnostics, Inc., 30 Spring Mill Dr., Malvern, PA 19355** *Avitech is an equal opportunity employer.*

AVITECH DIAGNOSTICS

The Scripps Research Institute

Ph.D. Programs in Chemistry and Macromolecular & Cellular Structure & Chemistry

The Scripps Research Institute will admit highly qualified students in the fall of 1996 to two Ph.D. programs: one integrating cell and molecular biology with structure and chemistry, and the other in chemistry.

Graduate studies at Scripps provide an exceptional training opportunity in a uniquely multidisciplinary environment with an emphasis on individualized training for each student.

Candidates must have a bachelor's degree and a strong background in biology and/or chemistry. Qualified applicants will be invited to visit the Institute. Financial support will be provided to all accepted students.

Since its establishment in 1961, the Institute has gained international recognition for its basic research in molecular biology, immunology, chemistry, neurosciences, cell biology, and plant biology.

Individuals interested in applying to either program should contact the Office of Graduate Studies for a brochure and application: **The Scripps Research Institute, Office of Graduate Studies, IMM 21, 10666 North Torrey Pines Road, La Jolla, CA 92037. Tel: (619) 554-8469. Fax: (619) 554-6602. Internet: http://www.scripps.edu.** The graduate programs are accredited by the Western Association of Schools and Colleges. TSRI is an equal opportunity institution.

UNIVERSITY OF ROCHESTER

Department of Anesthesiology

TAPS Program

Training Anesthesiologists as Physician Scientists

Combined Anesthesiology and Ph.D. Program

To prepare physician scientists for careers as independent investigators in an academic environment, the Department of Anesthesiology, University of Rochester Medical Center, offers a program to combine the anesthesiology residency or fellowship with research leading to a Ph.D. degree. The program allows qualified candidates to complete the requirements for a Ph.D. in a specific biomedical science while concurrently meeting the certification requirements of anesthesiology residency, or fellowship in anesthesiology specialties such as ambulatory anesthesia, pain management, cardiac, obstetrics, or critical care medicine.

Applicants must be U.S.A. permanent residents, have an M.D. (or D.O.) degree, or be enrolled in medical school, and meet the entrance requirements of both the Anesthesiology Residency program, and the graduate program of their choice. The Departments of Biophysics, Physiology, Pharmacology, and Neurobiology and Anatomy currently participate in the program; others will be added. The individualized curricula is designed by faculty mentors from the basic science and Anesthesiology departments to ensure a logical progression of both clinical and academic training.

Stipends and benefits are maintained throughout the period of training, commensurate with the participant's anesthesiology training post-graduate status. Candidates completing their residency during the program may also qualify as part-time staff anesthesiologists while finishing the requirements for the Ph.D. degree.

Further information and applications may be obtained from: Richard J. Rivers, M.D., Ph.D.; TAPS Program, Department of Anesthesiology; University of Rochester Medical Center; 601 Elmwood Avenue, Box 604; Rochester, NY 14642; Phone: 716-275-2143; FAX: 716-244-7271; E-mail: RRIVERS@ccmail.anes.rochester.edu

University of Rochester is an equal opportunity, affirmative action employer.

Nucleic Acids Chemist

Promega Corporation, a world leading biotechnology company, headquartered in one of America's most livable cities, Madison, WI, has an immediate opening for a senior research scientist to direct and manage the scientific efforts of a team dedicated to developing novel chemistries for the purification of nucleic acids.

Selected individual will identify and develop novel chemistries/technologies for the purification of DNA and RNA; manage strategic relationships for the purpose of expanding the nucleic acid purification business, and present research and technology development plans to senior management. Requirements include a Ph.D. in polymer chemistry, organic chemistry or inorganic chemistry and 5+ years of related industrial experience. Extensive knowledge of the binding interactions between macromolecules, including the physical forces and interactions involved in the binding of nucleic acids to sequence specific binding proteins and small molecular weight ligands such as antibiotics. Extensive experience in designing novel solid and solution phase extraction methods for macromolecules including DNA, RNA, lipids or proteins. Previous experience in the design of novel chemistries suitable for automated extraction/purification of macromolecules. Previous experience with current methods in molecular biology including nucleic acid purification, transfection, translations and probe labeling and hybridization techniques and automated robotics systems preferred.

Promega's mission is to become the most responsive supplier of biological reagents and systems in the worldwide biotechnology research products market. We strive to provide our employees with a rewarding work experience and to achieve unparalleled excellence, quality and prosperity in our business. Relocation assistance will be provided. To apply, send resume to: **Promega Corporation, Human Resources (11104S), P.O. Box 7879, Madison, WI 53707-7879.** For additional career information, call our Career Opportunities Line at (608) 277-2539. To learn more about our products and our company, visit our home page at http://www.promega.com. EOE

Promega

POSITIONS OPEN

OUTSTANDING OPPORTUNITIES FOR GRADUATE STUDENTS AND POSTDOCTORAL FELLOWS
Vascular Biology at the University of Virginia

The University of Virginia offers a multidisciplinary program in Vascular Biology emphasizing physiology, pharmacology, biophysics and cell biology of the vessel wall. Twenty-four investigators are participants in a training program with a long history of successful mentoring of both graduate students and postdoctoral fellows. Our particular strengths are in the areas of biophysics, cell signalling and microcirculation. Laboratory investigations are underway in cell-cell communication, cardiac, smooth muscle and endothelial cell electrophysiology, smooth muscle cell signal transduction and leukocyte-endothelial cell interactions. Details of the faculty interests, a description of the program, and application forms may be found on World Wide Web at **http://www.med.virginia.edu/inter-dis/cvrc/home.html.** Interested and competitive applicants may also apply directly to: **Dr. Brian R. Duling, Department of Molecular Physiology and Biological Physics, Box 449, Health Sciences Center, University of Virginia, Charlottesville, VA 22908.** *These positions are restricted to permanent residents or citizens of the United States. The University of Virginia is an Equal Opportunity Employer.*

POSTDOCTORAL POSITION
Mayo Clinic Scottsdale
Department of Biochemistry and Molecular Biology

POSTDOCTORAL POSITION focused on functional analysis of a carcinoma-associated transmembrane mucin MUC1. Project includes development and analysis of mammary gland tumors induced by chemicals and other means in Muc-1 null and wild type mice and analysis of phenotype of Muc-1 null mice using differential display or subtractive hybridization. Applicant should be experienced in molecular and tumor biology and immunology. Position available May 1996. Applicants should send curriculum vitae, three recommendation letters, and a one paragraph description of research interests to: **Dr. Sandra Gendler, Department of Biochemistry and Molecular Biology, Mayo Clinic Scottsdale, S. C. Johnson Medical Research Building, 13400 East Shea Boulevard, Scottsdale, AZ 85259.**

TWO POSTDOCTORAL POSITIONS
HARVARD MEDICAL SCHOOL

1) Position available to study the molecular mechanisms involved in neuronal apoptosis and Alzheimer's disease. Experience in neuronal cell culture and biochemistry is required. 2) Position available to study the regulation and function of the new Alzheimer's disease genes. Experience in molecular and cell biology is required. Send curriculum vitae, a brief description of research experience, and names of three references to: **Dr. Bruce Yankner, Children's Hospital, Enders 260, 300 Longwood Avenue, Boston, MA 02115. FAX: 617-738-1542.** *An Equal Opportunity/Affirmative Action Employer.*

POSTDOCTORAL POSITION available January 1, 1996 in digestive ecology/microbiology focusing on technologies for inhibition of methanogenesis and GI tract pathogen (*E. coli 0157:H7, Salmonella*) colonization. The successful candidate will have microbial physiology background with anaerobic expertise in methanogens, pathogens, with good molecular biology and writing skills. Salary $25,000, two years. Send curriculum vitae and names of references to: **Dr. F. M. Byers/Dr. S. C. Ricke, Institute of Food Science and Engineering, 120 Rosenthal, Texas A&M, College Station, TX 77843-2259. Email: fmbyers@acs.tamu.edu; FAX: 409-845-5292.**

POSTDOCTORAL POSITION available immediately to study molecular mechanisms underlying malignant transformation. A strong background in molecular and cell biology is required. Experience with PCR, immunohistochemistry, and antibody production are desirable. Send résumé and the names of three references with telephone numbers to: **Mark Dayton, M.D., Ph.D., Department of Medicine, Section of Hematology/Oncology, LSU Medical Center—Shreveport, 1501 Kings Highway, Shreveport, LA 71130.** *LSU Medical Center is an Equal Opportunity Educator and Employer.*

FOUR POSTDOCTORAL/PH.D. POSITIONS

A multi-university collaborative project examining the evolutionary ecology of exotic species introductions into the Great Lakes is looking for four candidates for postdoctoral or Ph.D. studies. Four research areas are currently under investigation: 1) Zooplankton diversity: allozyme and DNA markers will be used to diagnose unrecognized species invasions and to ascertain the source locations of currently recognized exotics. Contact: **P. D. N. Hebert; Email: phebert@uoguelph.ca.** 2) Theoretical ecology: mathematical theory of biological invasions. Contact: **P. Yodzis; Email: pyodzis@uoguelph.ca.** 3) Zooplankton feeding ecology: feeding rates and prey electivities, effects of *Bythotrephes* density on per capita feeding rates, and spatial distribution of this predator and its prey. Contact: **W. G. Sprules; Email: gsprules@cyclops.erin.utoronto.ca.** 4) Diffusion of exotic species: development of microsatellites for species of exotic molluscs and GIS modelling of diffusion from the source populations identified with these molecular markers. Contact: **E. G. Boulding; Email: boulding@uoguelph.ca.** Application packages should include a résumé, academic transcripts, the names, telephone numbers, and Email addresses of three academic referees, and an explanation of how your background relates to the position, and should be sent to: **Dr. Elizabeth Boulding, Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1 Canada.** Positions will remain open until they are filled. **D. K. Padilla** (U. Wisconsin, Madison) and **J. E. Havel** (Southwest Missouri State U.) are also collaborators on this project. *The University of Guelph is committed to an employment equity program that includes special measures to achieve diversity among its faculty and staff. We therefore particularly encourage applications from qualified aboriginal Canadians, persons with disabilities, members of visible minorities, and women.*

POSTDOCTORAL RESEARCH
UNIVERSITY OF ILLINOIS AT CHICAGO
COLLEGE OF MEDICINE
Molecular Biology and Functions of Eosinophils

POSTDOCTORAL POSITIONS available in laboratory moving to the Department of Biochemistry starting March/April 1996 for two Ph.D.'s: (1) to characterize the regulatory regions of eosinophil genes, to clone and characterize transcription factors that regulate eosinophil-specific gene expression, and to study the role of these factors in eosinophil development and function. Genes under study include the alpha subunit of the IL-5 receptor, eosinophil-generated cytokines, granule cationic proteins, and CLC protein. Molecular biology experience in gene regulation/transcription factor research desirable. (2) To analyze structure-function relationships for granule proteins/enzymes of eosinophils, specifically studying their mechanisms of action in the pathogenesis of inflammation, tissue damage and airways dysfunction in asthma, allergic and other eosinophil-associated diseases. Experience in structural biology, and protein engineering, expression, and purification desirable. Send your curriculum vitae, three reference letters, and two reprints to: **Steven J. Ackerman, Ph.D., Department of Medicine, RE219, Beth Israel Hospital and Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215.** *The University of Illinois is an Affirmative Action Equal Opportunity Employer.*

POSTDOCTORAL POSITION available to study the mechanism of action of G-protein oncogenes of the *RAS* family in transformation and mitogenic signaling (**Chan** *et al.*, *PNAS*, 91:7558–7562). Highly motivated individuals with strong training in biochemistry/molecular biology essential. Experience with cell culture techniques highly desirable. Send curriculum vitae and names of three references to: **Dr. Andrew M. L. Chan, The Derald H. Ruttenberg Cancer Center, The Mount Sinai Medical Center, 1230 York Avenue, Box 250, New York, NY 10021. Email: chanc@rockvax.rockefeller.edu; Telephone: 212-327-7343; FAX: 212-327-7342.**

POSTDOCTORAL POSITION available immediately to study molecular mechanisms underlying urological malignancies. A strong background in molecular and cell biology is required. Experience with immunhistochemistry and antibody production are a plus. Send résumé, names of three references (and their telephone numbers) to: **Oliver Sartor, M.D., Department of Urology, LSU Medical Center, 1501 King's Highway, Shreveport, LA 71130.** *The University is an Equal Opportunity/Affirmative Action Employer.*

Wayne State University

POSTDOCTORAL RESEARCH ASSOCIATE position in lung toxicology/cell biology available immediately to study cellular and subcellular events involved in the inflammatory and permeability changes in the airways following exposure to air pollutants. The studies utilize innovative *in vivo* and *in vitro* approaches to examine biological effects. Participation in studies aimed at analyzing cytokines, cell adhesion molecules, cytoskeleton, inflammation, and epithelial injury is expected. Experience can be drawn from immunocytochemical, morphological, and biochemical procedures. Send curriculum vitae to: **Deepak K. Bhalla, Ph.D., Department of Occupational and Environmental Health Sciences, College of Pharmacy and Allied Health Professions, 628 Shapero Hall, Wayne State University, Detroit, MI 48202.**
Wayne State University (WSU) is an Affirmative Action/Equal Opportunity Employer. WSU—People Working Together to Provide Quality Service. All buildings, structures and vehicles at WSU are smoke-free.

POSTDOCTORAL RESEARCH ASSOCIATE

The Ecosystems Center at the Marine Biological Laboratory has an opening beginning January 1, 1996. The project will trace the importance of nutrients and organic matter from the watershed to the oligohaline foodweb of an estuary by experimentally adding 15N-enriched dissolved inorganic nitrogen and following uptake in biota. Should have a Ph.D., with a strong background in higher trophic levels, food webs, and modeling plus good writing skills and the ability to work well in collaborative research. Deadline is December 22, 1995 or until a suitable candidate is identified. Position is funded for two years. Please send résumé, names of three references, general statement of interest, and copies of papers to: **Human Resources, Marine Biological Laboratory, Woods Hole, MA 02543. Telephone: 508-289-7422; Email: resume@mbl.edu.** *Equal Opportunity/Affirmative Action/Non-smoking workplace.*

POSTDOCTORAL POSITION to investigate the mechanism by which G proteins and their components regulate the activity of phospholipases via protein kinases/phosphatases in cells transfected with wild type and mutated G protein coupled receptors and signaling elements. Applicants must have a Ph.D., M.D., or D.V.M. degree with experience in the area of molecular biology. Please send curriculum vitae and the names of three references to: **Dr. K. U. Malik, Department of Pharmacology, University of Tennessee, Memphis, 874 Union Avenue, Memphis, TN 38163. Telephone: 901-448-6075; FAX: 901-448-7300.** *The University of Tennessee, Memphis is an Equal Employment Opportunity/Affirmative Action/Title VII/Title IX/Section 504/ADA Employer.*

POSTDOCTORAL POSITION. Available immediately to participate in NIH-supported research project on insulin regulation of gene expression at Case Western Reserve University School of Medicine. Studies use a multidisciplinary approach including interaction of proteins, generation of transgenic mice, and measurement of intermediary metabolism. Experience in *in vivo* metabolism/biochemistry and molecular methods is preferred. Send or FAX curriculum vitae and three references to: **Dr. Jacob E. Friedman, Department of Nutrition, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106. Telephone: 216-368-1616; FAX: 216-368-4544.**

POSTDOCTORAL POSITION is immediately available to study the regulation of eukaryotic transcription by hypoxia. The work will focus on the human erythropoietin gene and its complex regulation by oxygen and orphan receptors. The applicant must have a Ph.D. and experience in molecular biology. Send a description of your research interests and achievements, a curriculum vitae, and the names and addresses of three references to: **Kerry L. Blanchard, Ph.D., M.D., Louisiana State University Medical Center, Center for Excellence in Cancer Research, Treatment and Education, 1501 Kings Highway, Shreveport, LA 71130-3932. Email: kblanc.mailsh.lsumc.edu.** *Louisiana State University Medical Center is an Equal Opportunity Educator and Employer.*

MEDICAL COLLEGE OF WISCONSIN

Chairperson, Department of Cellular Biology and Anatomy

The Medical College of Wisconsin is reopening the search for a Chairperson for its Department of Cellular Biology and Anatomy.

The department is housed in a well-equipped modern facility. The faculty have productive research programs in developmental biology, visual sciences and neurosciences utilizing molecular, cellular, systems and imaging approaches. Departmental teaching responsibilities include the medical anatomy curriculum and the graduate program in cellular biology.

Candidates for chairperson should have a strong record of research achievements and scholarship, a commitment to graduate and medical education, and strong administrative skills to catalyze the continued growth of departmental programs. Candidates with research interests that include neurosciences, cancer, cardiovascular diseases are especially encouraged to apply although all outstanding candidates will be given serious consideration. The Medical College of Wisconsin has experienced a doubling in research funding during the last decade, and seeks to continue this trend through recruitment of excellent academic leadership and faculty.

Inquires should be directed to: Search Committee for Cellular Biology and Anatomy, c/o Office of The Dean, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.

Equal Opportunity/ Affirmative Action Employer M/F/H

St. Jude Children's Research Hospital

MOLECULAR NEUROSCIENCE

Department of Developmental Neurobiology

St. Jude Children's Research Hospital, a renowned center for biomedical research, has launched an initiative in the neurosciences housed in the recently completed Danny Thomas Research Tower. To this end, a new Department of Developmental Neurobiology, dedicated to the investigation of fundamental questions in contemporary neurobiology, has been created. The department is now recruiting postdoctoral fellows, graduate students and technical staff with research interests and training in molecular and cellular neurobiology. Currently, the department comprises Dr. Tom Curran (analysis of gene regulation; gene function during neurodevelopment), Dr. James Morgan (molecular basis of neuronal degeneration and regeneration; stimulus-transcription coupling in the nervous system), Dr. Suzanne Baker (molecular basis of carcinogenesis in Ewings sarcoma and pediatric brain tumors) and Dr. Michisuke Yuzaki (role of excitatory amino acid receptors in development and function of the nervous system; mechanisms of synaptic plasticity).

The campus provides a state-of-the-art research environment including new, custom designed laboratories and core facilities for molecular biology, peptide and protein chemistry, genomics and transgenie/knockout technology. The research towers presently house approximately 300 doctoral level scientists who provide a rich and interactive scientific environment. In addition, the Department is fostering strong intellectual and collaborative ties with neuroscientists and neurologists at the University of Tennessee Medical School. The faculty are affiliated with the Neuroscience Graduate Program in the Department of Anatomy and Neurobiology.

Postdoctoral candidates should send a CV and 3 letters of recommendation to the research lab of interest. Candidates for technical positions should write or call Kathleen Speck, human resources department (901-495-2770), for information and application forms. Potential graduate program. We expect to move into the new space in February, 1996; therefore, applicants should inquire before the end of January.

St. Jude Children's Research Hospital is an equal opportunity employment/affirmative action employer

NIH-FUNDED POSTDOCTORAL FELLOWSHIPS IN GAMETE AND EMBRYO BIOLOGY AT THE UNIVERSITY OF WISCONSIN, MADISON

Faculty and research topics are: **Barry Bavister**-fertilization and early development; **Bill Bement**-signal transduction/cytoskeleton; **Gary Borisy**-cell motility and the cytoskeleton; **Neal First**-maternal/embryonic transitions; **Ted Golos**-trophoblast determination; **Jeff Hardin**-gastrulation and morphogenesis; **John Hearn**-implantation; **Gary Lyons**-gene expression in embryonic stem cells; **John Parrish**-capacitation and acrosome reaction; **Gerald Schatten**-imaging motility during fertilization. *Candidates must be U.S. citizens or permanent residents.* Send curriculum vitae, three letters of recommendation, reprints/preprints, and a 2-3 page research proposal to: **Dr. Gerald Schatten, Gamete and Embryo Training Program, University of Wisconsin, 1117 West Johnson Street, Madison, WI 53706. FAX: (608) 262-7319. EMAIL:schatten@macc.wisc.edu** *An Affirmative Action/Equal Opportunity Employer.*

Senior Scientists

-Virology and Cell Biology

Quality Biotech Inc. is a leading international biotechnology service company engaged in contract research and biosafety testing for the biopharmaceutical industry. As part of our expansion we have openings for two Senior Scientists. The positions require a Ph.D. in virology, Microbiology, Cell Biology or Molecular Biology and postdoctoral training. Supervisory experience is a plus.

For the position in our Virology and Cell Biology Section we seek an individual who has experience with assays for human, animal/or insect viruses. The individual will supervise a number of established assays to test or characterize biopharmaceuticals, and establish new testing procedures in the areas of gene therapy, tissue engineering, and for other novel biopharmaceutical approaches. The individual should have experience with a wide variety of viruses and/or cell cultures. Experience with standard biosafety assays such as sterility, endotoxin and general safety tests is a plus.

A second position is available in our Viral Validation Group. This group is responsible for the design and implementation of customized protocols for validation of virus removal and inactivation by biopharmaceutical and blood product manufacturing processes. The Senior Scientist in this position will be responsible for development of mammalian virus assays and production of virus stocks and will be involved with a wide variety of purification procedures including chromatography, filtration, and various virus inactivation methods. The individual should have experience in virology, cell culture, and standard protein purification methods.

We offer health benefits, 401(k) plan and tuition reimbursement. Send resume and salary requirements to: Quality Biotech Inc., Human Resources, 1667 Davis Street, Camden, 08104. Equal opportunity employer.

Quality Biotech®
Biotechnology Testing Services

POSITIONS OPEN

DIRECTOR OF CANCER GENETICS

The Department of Medicine, Columbia University, is searching for a **PROFESSOR/ASSOCIATE PROFESSOR**—level candidate for an endowed chair with substantial resources to develop a basic research and related clinical program in cancer genetics. Interested individuals should send their curriculum vitae and bibliography to: **Dr. Karen Antman, Chief, Division of Medical Oncology, MHB 6N-435, 177 Fort Washington Avenue, New York, NY 10032.** *The University is particularly interested in qualified minority and women applicants. An Affirmative Action/Equal Opportunity Employer.*

ORGANIC PHYSICAL CHEMIST
Fluorescent Probe Synthesis

Position available for an individual with wide experience in synthetic organic chemistry of fluorescent probes. The research involves synthesis and testing of novel fluorescent probes for use in biochemistry, immunochemistry, and chemical/clinical sensing. Ph.D. required in organic chemistry, physical chemistry, or a closely related discipline. Experience with conjugatable probes, cation probes, and fluorescence spectroscopy necessary. Appointment at **RESEARCH ASSOCIATE** or **RESEARCH ASSISTANT PROFESSOR** level (non-tenure-track). Send curriculum vitae, three letters of recommendation, representative publications, and salary requirements to: **Probe Chemistry Search Committee, c/o Dr. J. R. Lakowicz, University of Maryland School of Medicine, Department of Biochemistry and Molecular Biology, 108 North Greene Street, Baltimore, MD 21201.** Application deadline: January 19, 1996. *Equal Opportunity Employer. Minorities and women are encouraged to apply.*

The USDA, Agricultural Research Service (ARS), Fargo, North Dakota, is seeking a **RESEARCH SCIENTIST** in animal physiology, chemistry, or biochemistry to conduct research on the metabolic fate of food or feed additives or environmental chemicals that may affect food safety. The research will seek to isolate and identify metabolites in mammalian tissues and excreta. The successful candidate is expected to develop a research program that will increase the understanding of physiological and biochemical mechanisms involved in the metabolism and disposition of food or feed additives or environmental chemicals that can be applied to the detection, prevention, and/or elimination of residues of these classes of compounds in animal products. Applicants must meet minimum education requirements as described in Vacancy Announcement ARS-D-6W-024. At least one year of research experience at the postdoctoral level in animal physiology (with a background in chemistry) would be required at the higher levels. Experience in mammalian surgery, metabolic studies (i.e., isolation and characterization of metabolites) and instrumentation (i.e., mass spectrometry, NMR, GLC, and HPLC) are advantageous. Salary ($34,295 to $49,989; plus 3.74% locality pay) is commensurate with experience. For information on application procedures call: **Ms. Melanie Nyquist, Administrative Officer, Telephone: 701-239-1203** for Vacancy Announcement Number ARS-D-6W-024 and for information on research project call: **Dr. Gerald L. Larsen, Research Leader, Telephone: 701-239-1231.** *ARS is an Equal Employment Opportunity Employer. U.S. citizenship is required.*

PLANT AND WETLAND ECOLOGIST

The Platte River Whooping Crane Trust, a non-profit organization that preserves habitat for migratory birds, is seeking a plant/wetland ecologist. Advanced degree; experience or education in wetland, riverine, or grassland ecology; strong communication skills; and commitment to conservation required. Expertise in wetland flora and restoration, hydrology, geomorphology, GIS, and applied natural resource management desirable. Responsibilities include land management, stewardship, and participation in water rights litigation. Send letter, résumé, salary history, and three references by December 30, 1995 to: **Dr. Paul Currier, Platte River Trust, 2550 Diers Avenue, Suite H, Grand Island, NE 68803. Telephone: 308-384-4633; FAX: 308-384-4634.**

RESEARCH ASSOCIATE IN ECOLOGY
Mountain Research Center
Montana State University, Bozeman

One-year position, beginning February 1996 (possible renewal for two more years). Research on biodiversity in montane environments (herpetological expertise preferred but not required); contribute to and facilitate interdisciplinary research on integration of human activities with natural ecosystem processes; help implement a multi-user biosystematics lab; mentor student research. Ph.D. in ecology or related field and record of publication in ecosystem processes required. The successful candidate will be able to integrate ecological and biosystematic techniques, attract extramural funds, and communicate effectively with other ecologists, biosystematists, GIS technical staff, land managers, economists, and sociologists. Submit curriculum vitae, three letters of recommendation, reprints of relevant publications, and a letter explaining relevant research interests to: **Research Ecologist Search Committee, Mountain Research Center, 408 Traphagen Hall, Montana State University, Bozeman, MT 59717.** Screening of applications begins on January 2, 1996, and continues until a suitable candidate has been chosen. Access **http://www.montana.edu/~wwwmrc/** for a detailed job listing.

ADA/Equal Opportunity/Affirmative Action/Veterans Preference.

CELL BIOLOGIST/BIOCHEMIST–Assistant professorship, tenure-track, anticipated fall 1996 for a broadly trained biologist to teach cell biology, biochemistry and participate in introductory biology. Ph.D. required.

Applicants must demonstrate excellence in undergraduate teaching and a research program that will involve undergraduates, including publication. Experience in small college setting desirable. Ursinus, northwest of Philadelphia in a suburban setting, is a highly selective, residential, co-ed liberal arts college of 1200 students with a long tradition of strength in science.

Send letter of application and curriculum vitae to: **Dr. Ellen M. Dawley, Department of Biology, Ursinus College, Box 1000, Collegeville, PA 19426-1000** by January 15, 1996. *Ursinus is an Equal Opportunity/Affirmative Action Employer.*

EXPERIENCED ELECTRON MICROSCOPIST

To participate in a highly interactive and motivated lab of molecular biologists; analysis of gene knockout mice with emphasis on the cytoskeleton. Ph.D. degree in pathology or cell biology is desirable. Long-term position.

Submit résumé and list of three references to:

Dr. Elaine Fuchs
Howard Hughes Medical Institute
The University of Chicago
5841 South Maryland Avenue MC 1028
Room N314
Chicago, IL 60637

The University of Chicago is an Affirmative Action/Equal Opportunity Employer.

NMR FACILITY MANAGER

For six Varian spectrometers (200-500 MHz) and a 4.7T imager/spectrometer. Responsible for calibration, maintenance, and cryogen service. Must be familiar with SUN computers and UNIX. Additional duties include operator training network and accounting management, implementation of new pulse sequences, and design and construction of specialized items such as surface coils. Experience with molecular modeling desirable. Ability to interact collaboratively with a variety of investigators is important. Ph.D. or equivalent experience desired. Send résumé and three letters of reference to: **Dr. Stanford Smith, Chemistry Department, University of Kentucky, Lexington, KY 40506-0055.** *The University of Kentucky is an Equal Opportunity Employer.*

MOLECULAR VIROLOGIST. Ph.D. with primary training and experience in virology. The individual sought for our research on neuroimmunology program will investigate neuropathogenesis of viral infections, such as HIV and herpes viruses, using *in vivo* and *in vitro* brain cell culture models. Familiarity with *in situ* hybridization and PCR analysis required. Competitive salary and excellent benefits package. Forward curriculum vitae to: **Minneapolis Medical Research Foundation, 914 South Eighth Street, 600 HFA Building, Minneapolis, MN 55404. Telephone: 612-347-5954.** *Equal Opportunity Employer/Affirmative Action.*

NUTRITIONAL BIOCHEMISTRY TECHNOLOGIST

Applicants should possess a Master's degree in nutrition with biochemical laboratory experience in a basic human research environment. In addition to hands-on experience with human nutritional biochemistry, the successful candidate should have a comprehensive background in sample processing protocols, methods development and validation, implementation of novel techniques, and instrument trouble-shooting and routine maintenance procedures. Data processing, data reduction, statistical analysis, and graphical presentation of analyzed data are components of the position. Excellent oral communication and technical writing skills are required. Previous experience with high performance liquid chromatography, enzyme linked immunosorbent assays, stable isotope measurements, radioimmunoassays, and/or spectrophotometry is highly desirable. Knowledge of requirements for standard laboratory safety and biohazardous materials handling is essential. The research conducted at the NASA Johnson Space Center includes definition of nutritional requirements for spaceflight, evaluation of protein, calcium, energy, and iron utilization as a function of spaceflight.

Interested applicants should submit current résumé, a list of references, and salary history **indicating position 95-F23-04** to: **KRUG Life Sciences Inc., Human Resources Department, P. O. Box 58827, Houston, TX 77258-8827.** *Employment Eligibility Verification Required. Equal Opportunity Employer. Minorities/Females/Disabled/Veterans. Smoke-free workplace.*

MICROBIOLOGIST

Hope College anticipates a three-year term appointment for a microbiologist beginning in August 1996. Appointment will be made at the **ASSISTANT PROFESSOR** level. The successful candidate will teach microbiology, another course appropriate to an undergraduate curriculum such as immunology, and be involved in the introductory course. In addition, the appointee will be expected to participate in one of the following ongoing research programs: cellular signaling–receptor cloning; molecular biology of lipid metabolism; induction of PR proteins in powdery mildew on barley; molecular biology of calcitrophic hormones; mechanisms of mutation in prokaryote and eukaryote cells; or molecular biology of parasite-associated cancers.

Hope College is a distinguished liberal arts college with an enrollment of 2,800 and a faculty of more than 180. Hope College maintains a strong tradition of "teaching through research" in the sciences. Hope College is affiliated with the Reformed Church in America.

Qualified applicants should arrange to have a curriculum vitae, transcripts, a statement of teaching philosophy and competencies, research interests, and three letters of recommendation forwarded to:

Dr. Paul Van Faasen, Chair
Department of Biology
Hope College
35 East 12th Street
Holland, MI 49422-9000

We will begin reviewing applications about January 10, 1996 and plan to make an appointment as soon as feasible thereafter.

Hope College complies with all legal requirements prohibiting discrimination in employment. Women and persons of color are strongly urged to apply.

PLANT TRANSFORMATION FACILITY MANAGER

The University of Nebraska is seeking an experienced individual to manage a new Plant Transformation Core Research Facility funded by the University's Center for Biotechnology. The position is a key component of an area of concentration aimed at generating novel useful transgenic plants. This person will oversee daily facility operations and collaborate with other project personnel. Target species are arabidopsis, tobacco, wheat, and soybean. Candidates should have a Ph.D. or M.S. with at least four years of experience in plant transformation. Closing date: January 15, 1996 or until suitable candidate is found. Résumé and three letters should be sent to: **Transformation Manager Search, N300 Beadle Center, P.O. Box 880665, Lincoln, NE 68588-0665.** Contact: **Dr. Paul Staswick, FAX: 402-472-7904; Email: pstaswick@crcvms.unl.edu.**

University of Nebraska is an Affirmative Action/Equal Employment Opportunity Employer.

FRENCH "ACADÉMIE DES SCIENCES" PROFESSORSHIPS

In order to foster international cooperation between academic and industrial research groups, the French Académie des Sciences, the Centre National de la Recherche Scientifique and Elf Aquitaine will open a limited number of one-year professorships for the 1996-1997 period. The positions are to be held in France at an academic institution or at an Elf laboratory or both.

These openings are reserved for research professionals at all levels, from Associate to Full Professor, and may be held in conjunction with a sabbatical leave.

The positions include generous benefits such as housing arrangements and travel expenses, and depend upon the candidate's experience. Selection criteria include the quality of research achievements and the relevance of the proposed program to Elf research activities, which cover the following domains:

-Earth Sciences, with emphasis on surface seismology, sedimentology, organic and mineral geochemistry, and the physics of hydrocarbon fluids, including their interactions with rocks.

-Chemical Sciences, with emphasis on polymers, halogen and thio-chemistry, catalysis, photo-chemistry and process engineering research.

-Environmental Sciences, with emphasis on the evolution of the geosphere and biosphere due to human activities and the methodology of biological indicators.

-Life Sciences, with emphasis on the biochemistry of receptors as applied to the central nervous system, and cardiovascular, thrombotic and degenerative diseases.

Prearranged agreements with French academic laboratories are eligible for these professorships and will be appreciated although not mandatory. Preference will also be given to candidates whose research programs include significant interaction with Elf R&D staff.

There are no citizenship restrictions. Mastery of French is not required, although some familiarity with French culture is expected to be helpful for the selected candidates.

Formal applications should include a detailed résumé, a description of the research project and any information relevant to the selection of the most qualified candidates. They should be sent to the following addresses before February 29, 1996.

-For North America-based candidates:

J. BODELLE, Vice President Research & Development
Elf Aquitaine, Inc.
1899 L Street, N.W., Suite 500
Washington, D.C. 20036

For other candidates:

C. JABLON, Chief Scientist
Elf Aquitaine, DRTE
Tour Elf, Cedex 45
92078 Paris - La Défense, FRANCE

Some discussion with the candidates about the program may then be suggested by the selection committee before the final announcements, which are scheduled for May 1996.

MIT

POSTDOCTORAL FELLOWSHIPS
Toxicology

Immediate openings for 2 NIH-sponsored postdoctoral fellowships *(U.S. citizenship or permanent residency required for both positions):*

1) To study the role of DNA conformation, chromatin structure, and nuclear architecture in targeting of oxidative DNA damage in cells. Prefer recent Ph.D. with experience in molecular biology, biochemistry, and antibody use/production. Send, FAX, or e-mail curriculum vitae, three letters of reference, and statement of skills/interests to: **Prof. Peter Dedon, Division of Toxicology, 16-336, MIT, Cambridge, MA 02139-4307; FAX: (617) 258-8676; e-mail: pcdedon@mit.edu.**

2) To study epithelial proliferation and apoptosis in inflammatory bowel disease. Experience with molecular biology methods and rodent models required. Familiarity with bacterial pathogenesis preferred. Send curriculum vitae, three letters of reference, and statement of skills/interests to: **Prof. David B. Schauer, Division of Toxicology, MIT, Room E18-564, Cambridge, MA 02139-4307.**

MIT is an Equal Opportunity/Affirmative Action Employer
MIT is a non-smoking environment

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

HARVARD MEDICAL SCHOOL
Department of Microbiology and Molecular Genetics

POSTDOCTORAL
RESEARCH FELLOW

Applications are invited from candidates to join two bacterial studies underway in the laboratory of Prof. E.C.C. Lin. (1) Physiological analysis and enzymatic characterization of the two-component signal transduction systems, Arc and Cpx, in *Escherichia coli*. (2) Elucidation of the anaerobic control of a regulon involved in glycerol fermentation in *Klebsiella pneumoniae* and monitoring evolutionary adaptations of the system in an *E. coli* host. Applicants should state their preference. The successful candidate should have a recent doctoral degree and significant experience in molecular genetics, bacterial physiology, and enzymology. The stipend will be commensurate with experience. Further enquires may be directed to Prof. Lin, Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115. FAX: (617) 738 7664; E-mail: elin@warren.med.harvard.edu Applications should be accompanied by c.v. and three letters of recommendation. *Harvard University is an equal opportunities employer.*

ALBERT EINSTEIN COLLEGE OF MEDICINE
OF YESHIVA UNIVERSITY

FACULTY POSITIONS

ALBERT EINSTEIN
COLLEGE OF MEDICINE (AECOM)

DEPARTMENT OF
MICROBIOLOGY
AND IMMUNOLOGY

Faculty positions are available on a tenure track for innovative investigators studying all aspects of immunology and the molecular pathogenesis of infectious organisms. Candidates can be at the beginning of their independent careers or more advanced in their academic pursuits. The Department has NIH Training Grants available to support pre- and postdoctoral students including MSTP students studying toward both the Ph.D. and M.D. degrees. AECOM provides core facilities including analytic ultrastructure analysis, production of transgenic and knockout mice, generation of hybridomas for monoclonal antibodies, FACS analysis, peptide synthesis and sequencing, and mass spectroscopy. There is a P3 facility available for handling infectious agents. Interested investigators should send their curriculum vitae, bibliography, a statement of research interests and 3 letters of recommendation to **Ms. Georgia Varoutis, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Bronx, New York 10461.** *An Equal Opportunity Employer.*

ALBERT EINSTEIN
COLLEGE OF MEDICINE
Toward the
advancement of medicine
...and your career

DIRECTOR
SHOALS MARINE LABORATORY
CORNELL UNIVERSITY

The Section of Ecology and Systematics invites nominations and applications for the Bartels Director of the Shoals Marine Laboratory (SML). The SML, a shared venture with the University of New Hampshire (UNH), is primarily an undergraduate teaching program located on Appledore Island in the Gulf of Maine. Although based at Cornell, the Director will be responsible for implementing the SML's academic programs and overseeing operations, will serve as liaison with the UNH, and will be involved with fundraising. A candidate with a distinguished record of research and teaching in marine sciences is sought; the area of specialization could range from the organismal to ecosystem level, with an emphasis in any area of ecology, evolutionary biology or systematics. The successful candidate should have a strong commitment to undergraduate education, administrative experience, and carry on an active research program. We especially seek applications from established scientists who would be appointed as Associate or Full Professor with tenure, at Cornell.

Letters of nomination should include the nominee's name, current position, address, tel/fax number(s) and e-mail address. **Applications** should include a curriculum vitae, copies of representative publications, a statement of research, teaching, and administrative interests and names of three referees, and be sent to: **Dr. Drew Harvell, Chair, Shoals Search Committee, Section of Ecology and Systematics, Corson Hall, Cornell University, Ithaca, New York 14853-2701.** (Public FAX: (607) 255-8088; E-mail: CDH5@CORNELL.EDU; World Wide Web: HTTP://WWW.SML.CORNELL.EDU). Screening of completed applications will begin February 5 and continue until the position is filled. *Cornell University is an Affirmative Action/Equal Opportunity Employer.*

CALIFORNIA STATE UNIVERSITY
SAN BERNARDINO

DEAN
SCHOOL OF NATURAL SCIENCES

California State University, San Bernardino invites nominations and applications for the position of Dean of the School of Natural Sciences. The preferred starting time is August 1, 1996. Reporting to the Vice President of Academic Affairs, the Dean is responsible for the academic and administrative leadership of nine departments within the School.

The Dean will be responsible for representing the School to the University and its external constituencies. Candidates should have an earned doctorate in one of the disciplines in the School of Natural Sciences and be eligible for a faculty appointment at the rank of Professor with tenure in one of the departments.

Compensation is competitive. Review of applicants will begin January 31, 1996 and continue until the position is filled. The applicant should submit a resume accompanied by the names, telephone, fax numbers and e-mail addresses of three references to: **Office of the Vice President for Academic Affairs, Chair, School of Natural Sciences Dean Search Committee, California State University, San Bernardino, 5500 University Parkway, San Bernardino, CA 92407.** *California State University San Bernardino is an Affirmative Action/Equal Opportunity Employer, and has a strong institutional commitment to the principle of diversity in all areas.* **For More Information see the School of Natural Sciences Home Page: http:\\www.csusb.edu**

ENVIRONMENTAL SCIENCES

The University of Southern California announces the formation of the **USC WRIGLEY INSTITUTE FOR ENVIRONMENTAL STUDIES**, which will complement ongoing environmental programs at USC. The current initial phase of the development of this institute includes renovation of USC's existing laboratories and teaching facilities on Santa Catalina Island (about 25 miles off our local coast) and the creation of the following two **ENDOWED** positions, for which we invite applications:

THE WRIGLEY CHAIR IN ENVIRONMENTAL STUDIES. This is a tenured faculty position in the field of **ENVIRONMENTAL BIOLOGY**. We seek an active researcher with a distinguished record whose research efforts would include marine or terrestrial work at Catalina Island, although his or her primary office and laboratory can be on the USC campus. Ideally, this individual would have a broad, global perspective, although preference will be given to scholars who can also take advantage of the special opportunities afforded by the Channel Islands' environments. The successful candidate will be expected to participate in our undergraduate and graduate educational programs.

DIRECTOR OF THE USC WRIGLEY INSTITUTE FOR ENVIRONMENTAL STUDIES. This individual is expected to lead in the development and implementation of activities at the Catalina laboratories, including scientific research, university courses, conferences, public education, and outreach. We are looking for an entrepreneurial individual with experience administering a field research station or similar facility. Residence at Catalina is expected. Scientific and teaching skills (preferably a Ph.D.) and the demonstrated ability to work constructively with scientists and government agencies are required. This position may carry an academic appointment.

Applicants should send a résumé, a cover letter describing their interests relating to the position, and the names, addresses, telephone/FAX numbers, and e-mail addresses of at least 3 references to:

Chair, Wrigley Search Committee
Department of Biological Sciences
University of Southern California
Los Angeles, CA 90089-0371

Applications will be considered beginning 15 January 1996, and will be accepted until the positions are filled.

USC is an Equal Opportunity / Affirmative Action Employer. We particularly encourage women and minorities to apply.

RESEARCH & DEVELOPMENT POSITIONS

Diagnostic Products Corporation, a leading independent and well-established immunodiagnostic company, is expanding its R&D and is seeking a broad range of scientific talent to assume key roles in that expansion. Opportunities have created an immediate need for individuals in the following departments.

PROTEIN CHEMISTRY

Creative individual with a Ph.D. or M.S. in Chemistry or Biochemistry with experience in Peptide/Protein Chemistry instruments. Strong background and demonstrated skill to employ various analytical techniques, especially Mass Spectrometry, for peptides and proteins.

MOLECULAR BIOLOGY

Immediate openings exist for Ph.D. Scientists trained in Molecular Biology and Microbiology, with experience in development of diagnostic assays using immunoassay and DNA probe technology.

HYBRIDOMA

Ph.D. Scientists with experience in hybridoma technology, radioimmunoassay development and cellular immunology.

IMMUNOASSAY DEVELOPMENT

Ph.D. or M.S. in Chemistry, Biochemistry or related field with 2+ years of industrial experience in immunodiagnostic industry. Strong background in solid phase protein immobilization and assay development and thorough knowledge in theory of immunoassays are essentials.

We offer competitive salary, an attractive benefits package, and the opportunity to make a significant contribution to the immunodiagnostic field. Please send your resume and salary history to:

Human Resources
Diagnostic Products Corporation
5700 W. 96th Street
Los Angeles, CA 90045

IMMUNOLOGIST

Cytel Corporation is a publicly-held biotechnology company whose headquarters are located in San Diego, CA. We are committed to the discovery, design and development of immunotherapeutics for the treatment of serious human diseases. We are seeking a talented cellular immunologist to be a part of a team at Cytel's new facility in Charlottesville, Virginia. If you wish to contribute to carrying out original and applied research in the areas of antigen identification and peptide sequencing by mass spectrometry, please consider joining our Charlottesville research team.

The individual we are seeking will be a critical part of a team-oriented environment who will be responsible for culturing human CD8 T-cells, TIL and tumor cells, bioassay of T-cell activation, and MHC purification and peptide extraction. The selected individual must be familiar with peptides as well as basic molecular biology techniques, DNA transfections, and SDS-PAGE.

Candidates must have a Ph.D. in immunology or related scientific discipline plus a minimum of 3-5 years of post-doctoral experience as well as a proven record of scientific accomplishment and/or antigen identification. Individual must have the ability to work with other team members who have expertise in analytical biochemistry and mass spectrometry. Excellent verbal and written communication skills are essential as well as being self-motivated.

Cytel offers a challenging environment, competitive salary and benefit programs, as well as stock options and an employee stock purchase plan. Qualified candidates please send your curriculum vitae to attention of Job Code:CE44/190, Cytel Corporation, 3525 John Hopkins Court, San Diego, CA 92121-1121. We are an equal opportunity employer.

CYTEL

MIT

Faculty Position
Department of Brain & Cognitive Sciences

The MIT Department of Brain and Cognitive Sciences anticipates making a tenure-track appointment in experimental cognitive science at the Assistant Professor level. Candidates should have an active research interest in one or more of the following areas: memory, high-level vision, cognitive development, reasoning. skill acquisition, and attention. Responsibilities include graduate and undergraduate teaching and research supervision.

Applications should include a brief cover letter stating the candidate's research and teaching interests, a *vita,* three letters of recommendation and representative reprints. Send applications by **February 1, 1996** to: **Cognitive Search Committee, Dept. of Brain & Cognitive Sciences, E25-406, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139-4307.**

Qualified women and minority candidates are especially encouraged to apply.

MIT is an Affirmative Action/Equal Opportunity Employer.

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

PHARMACOKINETIC SCIENTISTS

Applications are invited for two Pharmacokinetic Scientist positions in the Department of Pharmacokinetics and Metabolism. Your primary responsibilities will include supporting preclinical and clinical development of recombinant proteins and small molecule therapeutics by designing, conducting, analyzing, and reporting pharmacokinetic/pharmacodynamic studies.

To qualify, you should be a scientist with doctoral level training in pharmacokinetics, pharmaceutical sciences or a related discipline and 2+ years of industrial or academic experience in pharmacokinetics. Excellent scientific, interpersonal and oral/written communication skills as well as the ability to work in a team environment are essential. Experience in the drug development process, regulatory submissions, and demonstrated capabilities in advanced pharmacokinetic data analysis are plusses.

Genentech is an international biotechnology company that discovers, manufactures and markets human pharmaceuticals for significant unmet medical needs. We offer an excellent salary and benefits package to full time employees that includes fully paid medical/dental/vision coverage, free health club membership, and Company stock purchase opportunities. For immediate consideration, please send your resume to Genentech, Inc., Human Resources Dept. NSDB, 460 Pt. San Bruno Blvd., South San Francisco, CA 94080. Genentech is an Equal Opportunity Employer. We value the contributions of our diverse workforce.

Genentech, Inc.

Postdoctoral Fellowship Program

The Boston Biomedical Research Institute (BBRI) invites competitive applications for postdoctoral research positions in the laboratory of a BBRI faculty member under the BBRI Scholars Program. BBRI is an independent biomedical research center that offers an exceptionally collegial and intellectually stimulating environment. Current research programs at BBRI include:

Leonard Adam	Signal transduction mechanisms regulating vascular smooth muscle contractility
John Badwey	Biochemical basis of cell stimulation and transmembrane signalling
John Codington	Glycoproteins of cancer cells and their use in diagnosis and treatment of cancer
Peter Coleman	Protein prenylation enzymes and cell proliferation; mitochondrial bioenergetics
John Gergely	Mechanism of muscle contraction and its regulations Biochemical and biophysical studies
Zenon Grabarek	Structure and function of calcium binding proteins
Philip Graceffa	Control of smooth muscle contraction: Structure and function of tropomyosin and caldesmon
Jenshiang Hong	Mechanisms of gene activation in bacterial signal transduction
Noriaki Ikemoto	Molecular mechanism of excitation-contraction coupling
Saroj Joshi	Energy coupling mechanisms in mitochondrial protein pumping ATPase
Sherwin Lehrer	Regulation of muscle contraction: Spectroscopic and chemical studies
Enne Lu	Function-structure relation of contractile proteins
Kathleen Morgan	Subcellular mechanisms of contraction and growth of smooth muscle
Henry Paulus	Complex biosynthetic pathways: Control of enzyme activity and gene expression
Victor Raso	Immunological approaches to the design of new therapies for cancer and AIDS
Nillma Sarkar	Structure, function and biosynthesis of bacterial poly(A) RNA; chromosome replication
Michael Sherman	Molecular chaperones; control of the cell cycle
Walter Stafford	Study of protein and nucleic acid interactions by analytical ultracentrifugation
Terrence Tao	Structure and function of regulatory proteins in skeletal and smooth muscles
Vladimir Volloch	Organization, structure, and expression of eukaryotic genes
Albert Wang	Physical biochemistry of Ca-binding proteins and chair targets in smooth muscle cells
Hartmut Wohlrab	Structure and function of integral membrane transport proteins; biochemistry of senescence

Applicants should submit a brief statement of research interests and curriculum vitae and arrange to have two letters of reference sent to: **Ms. Barbara Zillman, Administrative Assistant, Boston Biomedical Research Institute, 20 Staniford Street, Boston, MA 02114**. *Boston Biomedical Research Institute is an Equal Opportunity Employer.*

NEUROSCIENTISTS

The University of Calgary Neuroscience Research Group invites applications for two full-time academic neuroscientists at the assistant professor level or higher. These positions offer an excellent opportunity to develop an independent research program and collaborative interactions within a multidisciplinary research environment. Duties will include teaching and graduate student supervision. Positions are available in:

- molecular and/or cellular studies of the development or plasticity of the nervous system;
- cellular or systems neuroscience.

Qualifications include a PhD (or equivalent) and at least two years of postdoctoral experience.

The selected candidates must compete successfully for salary and establishment funding from the Alberta Heritage Foundation for Medical Research and/or Medical Research Council of Canada, and will have 75% of time protected for research. Interested persons are invited to visit our web site.

http://www.cns.ucalgary.ca/nrg/nrg.html

In accordance with Canadian immigration requirements, priority will be given to Canadian citizens and permanent residents of Canada. The University of Calgary is committed to Employment Equity.

Please submit a curriculum vitae and a statement of research interests, and arrange to have three letters of reference sent directly, by January 31, 1996 to:

Dr. S. Barnes, Chair
Neuroscience Research Group
The University of Calgary
3330 Hospital Drive N.W.
Calgary, Alberta T2N 4N1

THE UNIVERSITY OF CALGARY

CHAIR
DEPARTMENT OF PHARMACEUTICAL SCIENCES
SCHOOL OF PHARMACY
UNIVERSITY OF MARYLAND AT BALTIMORE

The University of Maryland at Baltimore [UMAB] School of Pharmacy invites applications and nominations of outstanding leaders and scholars in the pharmaceutical sciences for the position of Chair, Department of Pharmaceutical Sciences. Located in Baltimore's University Center just blocks from the Inner Harbor, UMAB is the professional campus of the University of Maryland and consists of the Schools of Pharmacy, Medicine, Dentistry, Nursing, Law, and Social Work. The University of Maryland Medical System, Baltimore VA Medical Center, and Medical Biotechnology Institute combine with the professional schools to make UMAB the fourth fastest growing research center in the country, with over one billion dollars of new building in the past decade. The School of Pharmacy has enjoyed an increase in its state budget and laboratory space over the past three years. These developments have contributed to the efficient implementation of the School's innovative all-Pharm.D. professional degree program and significantly increased our emphasis on research and graduate education.

The Department of Pharmaceutical Sciences, consisting of the broad disciplines of biomedicinal chemistry, pharmaceutics, and pharmacology, has in recent years grown dramatically. The Department now has 30 full-time faculty members, 10 of whom were hired into new positions within the past three years, and 75 graduate students. Extramural funding for research is currently in excess of four million dollars and ongoing investigations include collaborative projects with other researchers on campus and at the FDA, NIH, Johns Hopkins University, Walter Reed Army Institute of Research, and the pharmaceutical industry.

We are seeking a chair who has strong interpersonal and leadership skills, the ability to foster collaborative and interdisciplinary initiatives, and a commitment to continue the development of a diverse group of productive faculty into an outstanding department in both research and education. The successful candidate will be an excellent scientist with demonstrated management, teaching, and grantsmanship/fundraising skills.

An advanced degree in the pharmaceutical or related sciences and experience in pharmaceutical education are essential; a professional degree in pharmacy is desirable. Candidates are expected to have credentials sufficient to be appointed at the tenured full professor level. Salary is dependent upon qualifications and experience. Candidate screening will begin in January and continue until the position is filled.

Direct nominations or applications with a letter of interest, curriculum vitae, and names, addresses and telephone numbers of at least three references to:

Dr. George E. Dukes, Chair
Pharmaceutical Sciences Department Chair Search Committee
School of Pharmacy
University of Maryland at Baltimore
100 Penn St., Suite 205
Baltimore, Maryland 21201
(410) 706-7338

The University of Maryland at Baltimore is an Equal Opportunity/Affirmative Action/ADA Employer. Minorities and women are encouraged to apply.

The John P. Robarts RESEARCH INSTITUTE

RESEARCH SCIENTISTS

The Neurodegeneration Research Group at The Robarts Research Institute is comprised of a multi-disciplinary team of eight researchers whose interests are directed towards understanding the cellular and molecular mechanisms involved in development, death and regeneration within the nervous system. We are seeking to hire **two scientists** (Ph.D. and/or M.D.) whose work is focused on molecular mechanisms underlying neuronal death, cell signalling and transduction, or gene expression and transcriptional regulation. Priority will be given to investigators with highly molecular methods including genetic and transgenic approaches. The position and salary offered will be commensurate with experience. Establishment funds will be available for the first three years. Academic rank will be determined by cross appointments with the appropriate department at The University of Western Ontario. The John P. Robarts Research Institute, located on the attractive campus setting of The University of Western Ontario in London, Ontario, is an autonomous, modern facility that aptly reflects the strong growth and commitment to research in Southwestern Ontario. Qualified candidates should forward a curriculum vitae, an outline of research interests and scientific goals, reprints of key publications and a list of three references to: **DR. LYNNE C. WEAVER, DIRECTOR, NEURODEGENERATION RESEARCH GROUP, THE JOHN P. ROBARTS RESEARCH INSTITUTE, P.O. BOX 5015, 100 PERTH DRIVE, LONDON, ONTARIO, N6A 5K8 CANADA.** *In accordance with Canadian Immigration requirements, this advertisement is directed to Canadian citizens and permanent residents of Canada.*

DEAN, COLLEGE OF LIFE SCIENCES & AGRICULTURE
UNIVERSITY OF NEW HAMPSHIRE
DURHAM, NEW HAMPSHIRE

The University of New Hampshire is seeking a Dean of the College of Life Sciences and Agriculture to assume duties on July 1, 1996.

The University is a Land Grant, Sea Grant, and Space Grant university of some 12,400 students committed to providing comprehensive high quality undergraduate and graduate programs and to the promotion of excellence in teaching, scholarship, and public service. It is the only comprehensive research university within the University System of New Hampshire.

The College of Life Sciences and Agriculture is one of the University's five schools and colleges, with 1,730 undergraduate majors, 460 associate degree students, and 129 M.S. and 84 Ph.D. students. Its Dean is responsible for the administration of the College's academic programs in biological sciences, natural resources, agriculture, and social sciences; its interdisciplinary biology and genetics programs; and affiliated research centers. As the College's chief administrator, this individual reports to the Provost and actively participates in university governance as a member of the Dean's Council and the Academic Senate.

The Dean oversees eight academic units: the Thompson School of Applied Science and the Departments of Animal and Nutritional Sciences, Biochemistry and Molecular Biology, Microbiology, Natural Resources, Plant Biology, Resource Economics and Development, and Zoology. The position also carries responsibilities as Director of the New Hampshire Agricultural Experiment Station. The college mission is supported by about 104 full-time faculty, most of whom have appointments split between teaching and the Agricultural Experiment Station or UNH Cooperative Extension. The Dean is involved in interactions with several on-campus research centers, including the Institute for the Study of Earth, Oceans, and Space; the Environmental Research Group; the Marine Program Board; and the Water Resources Research Center.

The successful candidate will have a demonstrated commitment to undergraduate and graduate education and research in biological sciences, natural resources, agriculture, and social sciences. He or she will have had successful administrative experience in higher education including personnel and program management and evaluation, curriculum and program development, budget development and management, and academic planning. The Dean is expected to have the ability to act as an advocate for the College's programs; consult with faculty, faciliate undergraduate and graduate education, research, outreach and development; guide and promote faculty development in teaching and scholarship; and advance the College's affirmative action goals. The candidate's qualifications must include an earned doctorate and a record of teaching and scholarship appropriate to an appointment as a tenured professor in one of the College's departments.

Applications should include a brief statement of philosophy regarding the position, a curriculum vitae and the names and addresses of three references (who will not be contacted until the later stages of the search), and should be addressed to **College of Life Sciences and Agriculture Dean Search Committee, Office of Academic Affairs, Thompson Hall, University of New Hampshire, Durham, NH 03824.** Applications will be reviewed starting **January 26, 1996**, but the search will remain open until the position is filled.

The University of New Hampshire is an AA/EEO Employer. Women and minority candidates are encouraged to apply.

CORE FACILITY (MACROMOLECULAR SEQUENCING & SYNTHESIS) AT HUNTER COLLEGE SEEKS MANAGER

at the Research Associate level (Ph.D. in biomedical science or chemistry preferred, but other qualified applicants will be considered). Requires good knowledge of organic chemistry and liquid-phase protein sequencing. Experience with oligonucleotide synthesis, peptide synthesis, and HPLC preferred. Knowledge of DOS, Windows, Unix and Netware desired. The position requires close consultation with 35 faculty and students and supervision of one technician. Salary for this permanent position ($50,000 to $60,000) depends on qualifications. Attractive benefits. CV and references to: **Dr. T. Schmidt-Glenewinkel, Dept. of Bio. Sci., HUNTER COLLEGE, 695 Park Avenue, New York, NY 10021** EOE/AAE/AWDAE

DIRECTOR
Biotechnology Laboratory
The University of British Columbia

The Biotechnology Laboratory at the University of British Columbia was established in 1987 to catalyze the interdisciplinary development of Biotechnology at .B.C. The laboratory now comprises 13 independent faculty members with a strong record in attracting external funding. It includes an efficient administrative support system, excellent core facilities for molecular biology, a bioprocessing unit, and a teaching laboratory. The University is now searching for a distinguished scholar to serve as a new Director **commencing July 1, 1996,** or soon thereafter.

The candidate should be an internationally recognized scientist who will provide leadership, direction and vision to the Biotechnology Laboratory. The candidate will possess effective interpersonal and administrative skills and the ability to maintain strong, positive interactions with other academic units on campus and with the private biotechnology sector. The candidate should be willing to participate in fund-raising activities.

The new Director will lead the continued development of the Biotechnology Laboratory and will be provided with generous laboratory space, administrative support staff and facilities. The new Director will also hold an academic appointment in an appropriate Department.

In accordance with Canadian immigration requirements, priority will be given to Canadian citizens and permanent residents of Canada. UBC welcomes all qualified applicants, especially women, aboriginal people, visible minorities and persons with disabilities. The position is subject to final budgetary approval.

Applications or nominations for the directorship must be received **prior to February 15, 1996** and should include the curriculum vitae of the candidate, together with the names of three references, and be sent to:

UBC

Search Committee
Biotechnology Laboratory Directorship
c/o Dr. Barry C. McBride
Dean of Science
6270 University Boulevard, Room 1505
Vancouver, B.C. CANADA V6T 1Z4

INFLAMMATION PHARMACOLOGY
Investigator/Sr. Investigator

SmithKline Beecham Pharmaceuticals, a world leader in pharmaceutical research, has an opportunity for an Investigator to join the Department of Inflammation Pharmacology.

The selected candidate will participate in the drug discovery process for immunologically driven inflammatory diseases and identify new molecular targets for immunological diseases. Acting as a departmental resource in the field of Cellular and Molecular Immunology, the Investigator will also be required to establish new model systems/assays to facilitate research efforts, present findings at scientific meetings and publish in scientific journals.

Qualifications include a PhD (or equivalent) in Immunology, Molecular Biology or Cell Biology, and 2+ years of post-doctoral experience in immunology, immunopharmacology or related field. Candidates must also demonstrate expertise with state-of-the-art cellular and molecular immunology methodologies, as well as knowledge of T and B lymphocyte biology and cell-mediated immunity. The ability to take a creative approach to independent projects is equally essential.

Located in our technologically advanced research facility in suburban Philadelphia, SmithKline Beecham offers an excellent compensation/benefits/relocation package. Interested candidates should send CV and salary requirements to: SmithKline Beecham Pharmaceuticals, Job Code H-IPDG, P.O. Box 2645, Bala Cynwyd, PA 19004. We are an Equal Opportunity Employer, M/F/D/V.

SB
SmithKline Beecham
Pharmaceuticals
Challenging the natural limits.

Postdoctoral Fellows
Molecular & Cell Biology

The Lindsley F. Kimball Research Institute, a division of the New York Blood Center, invites applications for post-doctoral Fellow positions in the general area of molecular and cell biology of blood group antigens.

The fellows will participate in a collaborative, NIH-funded, SCOR program on Transfusion Biology and Medicine aimed at understanding the molecular basis of human blood groups in order to enhance the capabilities of blood banking services, characterize structure/function relationships and explore relations in disease processes. Candidates must offer a Ph.D. or MD degree supported by a s trong background in biochemistry and molecular biology, including experience in gene isolation and expression and recombinant technology.

The program project leaders include:

CHENG-HAN HUANG, MD, PH.D.
Molecular genetics of the red cell Rh antigens

COLVIN M. REDMAN, PH.D.
The Kell blood group system and the McLeod phenotype

A. OSCAR POGO, MD
The Duffy blood group protein

MARION E. REID, PH.D.
Molecular biology in clinical immunohematology

These positions will be available January, 1996 and command a competitive salary and excellent comprehensive benefits package. The Lindsley F. Kimball Research Institute comprises 17 laboratories carrying out autonomous research programs in diverse field of biomedical research. Interested candidates should send C.V. and the names of (3) references to: Colvin M. Redman, Ph.D., The New York Blood Center, 310 East 67th Street, New York, NY 10021. An Equal Opportunity Employer M/F/D/V.

Lindsley F. Kimball
Research Institute
A Division of the New York Blood Center

IMMUNOLOGIST

The University of Calgary Immunology Research Group invites applications for a full-time academic position in immunology at the Assistant Professor level or higher. This position offers an excellent opportunity to develop an independent research program and collaboration with individuals studying leukocyte-endothelial cell interactions *in vivo*, and various aspects of lymphocyte and neutrophil development and function.

Qualifications include a PhD and/or MD, at least two years of postdoctoral experience, and demonstrated excellence and productivity in the area of leukocyte adhesion under normal and disease states. Applications are encouraged from those using molecular and biochemical techniques to study function and/or structure of adhesion molecules.

The selected candidate must compete successfully for salary support and establishment funding from the Alberta Heritage Foundation for Medical Research and/or the Medical Research Council of Canada, and will have 75% of time protected for research. More information available at website: (http//:www.im.ucalgary.ca/)

In accordance with Canadian immigration requirements, priority will be given to Canadian citizens and permanent residents of Canada. The University of Calgary is committed to Employment Equity.

Please submit a curriculum vitae, a statement of aims and research interests, and reprints of two recent publications, and the names/addresses of three referees by January 15, 1996, to:

Dr. J.D. Reynolds, Chair
Immunology Research Group
The University of Calgary
3330 Hospital Drive N.W.
Calgary, Alberta, Canada T2N 4N1

THE UNIVERSITY OF CALGARY

SYMPOSIUM

The William Harvey
Medical Research Foundation
a Not-For-Profit Organisation

presents an international symposium on

New targets in inflammation: inhibitors of COX-2 or adhesion molecules

Monday 15th -Tuesday 16th April 1996
to be held at
Sheraton New Orleans Hotel
New Orleans, USA

Conference Chairmen
Nicolas Bazan and Sir John Vane

Topics include

- Structure of cyclo-oxygenases [COX-1 and COX-2]
- Induction of COX-2 in inflammation
- Differential inhibition of COX- 1 and COX-2 by NSAIDs
- COX-2 and COX-1 knockout and overexpression
- Development of selective COX-2 inhibitors
- COX-2 expression in different organs including uterus, brain and synovial tissue
- Toxicity of NSAIDs including renal side effects
- Leukointegrins and ICAM-ligands
- Cytokines and adhesion molecules in the inflammatory response

Faculty includes

NG Bazan [New Orleans] • P Cuatrecasas [Ann Arbor] •L Crofford [Ann Arbor] • D De Witt [East Lansing] •R DuBois [Nashville] • S Ferreira [Brazil] • G Fitzgerald [Philadelphia] • AW Ford-Hutchinson [Dorval] • WM Gallatin [Bothell] •H Jick [Lexington] • P Lipskey [Dallas] • P Loll [Philadelphia] •SG Morham [Chapel Hill] • J Oates [Nashville] • M Pairet [Biberach] • JR Vane [London] and PA Ward [Ann Arbor]

Poster presentations

Abstracts are invited

Hosted by

Louisiana State University
Neuroscience Center of Excellence, New Orleans, USA

For information

Apply to Dr. J Maclagan, Conference Secretariat, William Harvey Research Conferences, The Medical College, Charterhouse Square, London EC1M 6BQ, UK. Tel +44 171-982-6181; FAX +44 171-982-6094 or Mrs. Zevenaar Flowers-Davis, LSU Neuroscience Center, 2020 Gravier Street, Suite B, New Orleans, Louisiana 70112. Tel 504-568-6700, ext. 321; FAX 504-568-5801

CONFERENCE

8th International Congress

MOLECULAR PLANT-MICROBE INTERACTIONS

July 14-19, 1996 • Knoxville, Tennessee USA

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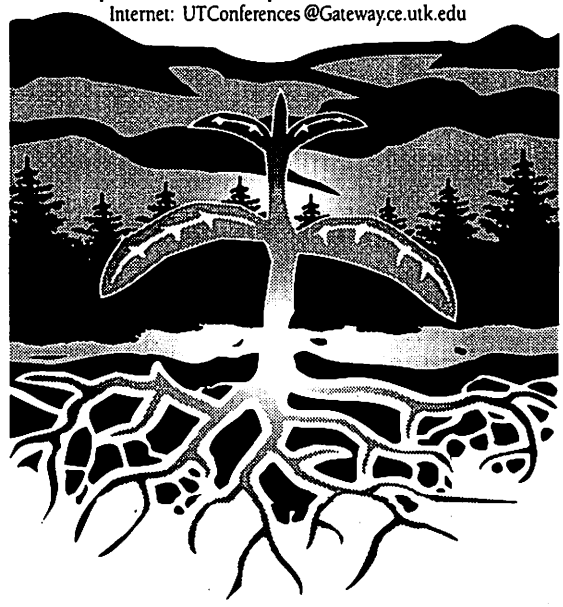
Phone: (423) 974-0280 • Fax: (423) 974-0264
WWW: http//www.ce.utk.edu/departments/conferences/confhome.html
Internet: UTConferences @Gateway.ce.utk.edu



MEETINGS

American Physiological Society

Physiology InFocus

Genetic Strategies for Understanding the Physiological Basis of Disease
Experimental Biology '96
April 14-17, 1996
Washington, DC

Monday, April 15 AM
Genetic Tools for Physiological Studies
Organizer: Garry R. Cutting
Speakers: Francis Collins, Mark Boguski, Phil Hieter, Barry Ganetzky

Monday, April 15 PM
Transgenic Strategies for Physiological Studies
Organizer: Craig Malbon
Speakers: Susumu Tonegawa, Craig Malbon, Ronald Kahn, Alexandra Joyner

Tuesday, April 16 PM
Genetic Diseases Affecting Ion Channels
Organizer: Raymond A. Frizzell
Speakers: Eric Hoffman, Thomas Jentsch, Kurt Beam, David MacLennan, Richard Boucher

Wednesday, April 17 AM
Ion Sensors and Transporters in Health and Disease
Organizer: Steven C. Hebert
Speakers: Edward Brown, Michael P. Kavanaugh, Jeffrey D. Rothstein, Matthias Hediger

For information, contact FASEB OSMC, Tel: 301-530-7010, Fax: 301-530-7014, E-Mail: eb@nsl.faseb.org

ASSISTANT PROFESSOR
ENVIRONMENTAL TOXICOLOGY
Biochemical/Physiological Toxicology
University of California, Santa Cruz

The Department of Biology at the University of California, Santa Cruz invites applications for a tenure track position at the level of Assistant Professor. The successful candidate will be expected to develop an extramurally funded research program in areas of biochemical and/or physiological toxicology focusing on fundamental molecular, biochemical or physiological mechanisms involved in the adaptations and responses of animals, plants, or microorganisms to environmental toxins. The candidate will be expected to teach both undergraduate and graduate courses in that area, and to actively participate in the development of a new graduate program in Environmental Toxicology. The successful candidate, who initially will be a member of the Department of Biology, will be the third of several appointments in a proposed Environmental Toxicology Program.

By January 31, 1996, applicants should submit a curriculum vitae, a brief description of research and teaching interests, copies of reprints, and arrange for three letters of recommendation to be sent to:

Biology/Toxicology Search Committee
Department of Biology
225 Sinsheimer Laboratories
University of California
Santa Cruz, CA 95064

Please refer to Provision #479 in your reply.

The University of California, Santa Cruz is an Equal Opportunity/Affirmative Action Employer.

Faculty Positions in Cancer Biology/Molecular Oncology

The newly formed Cancer Biology Program of Beth Israel Hospital is seeking applications for the position of Assistant Professor of Medicine. The successful candidate will hold the Ph.D. and/or M.D. degrees, and will have completed a minimum of two years of postdoctoral training in molecular or cellular biology, or genetics. Appointees will be expected to establish an independently funded research program in cancer biology. Applications are particularly encouraged from individuals with research interests and experience in the areas of (1) molecular genetic approaches (including knockout technology) to signal transduction with applications to oncology; (2) epithelial cell biology; or (3) novel targets for cancer therapy including apoptosis, cell cycle, repair/recombination, immunotherapy, telomerases. Generous start-up funds and some ongoing support will be provided. Members of the Cancer Biology Program currently focus on tyrosine phosphatases, steroid-thyroid hormone receptors, genetic instability in cancer and hematopoiesis. Candidates should send CVs and names of three references to:

Benjamin G. Neel, M.D., Ph.D.
Faculty Search Committee
Molecular Medicine Unit
Beth Israel Hospital, RW663
330 Brookline Avenue
Boston, MA 02215

The Beth Israel Hospital is an Equal Opportunity employer.

MOLECULAR
REPRODUCTIVE BIOLOGY/
DEVELOPMENTAL BIOLOGY

The Department of Physiology at the **University of Maryland School of Medicine** invites applications for a full-time, tenure-track position at the **Assistant Professor level in the area of Molecular Reproductive/Developmental Biology. We** are specifically seeking candidates who use innovative cellular, molecular, genetic, and/or transgenic approaches to study the regulation of cell migration, proliferation, differentiation, and related developmental processes in tissues of the reproductive system. Of particular interest are the signalling pathways through which hormones, growth factors, and other mediators control these complex events. Applicants must have a Ph.D. or M.D., at least two years of postdoctoral experience, and the potential to develop a strong, independent research program. Teaching responsibilities include participation in medical and graduate courses. The Department of Physiology ranks near the top of physiology programs in the U.S. in NIH-funded research and citations per faculty member. The Department and School of Medicine, provide a stimulating environment for research, with several interdisciplinary research and training programs - including Reproductive Biology, Molecular and Cell Biology, Membrane Biology, and Neuroscience - that promote interaction between faculty, fellows, and graduate students. Three NIH training grants provide core support for the strong graduate training program. The position is highly competitive with regard to start-up funds, laboratory space, salary, and benefits. Applicants should submit (1) curriculum vitae, (2) concise statement of research experience and plans, and (3) names of three references to: **Robert D. Koos, Ph.D., Chair, Molecular Reproductive Biology Search Committee, Dept. of Physiology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201-1559; Tel. 410/706-8033.** Review of applications will begin January 8, 1996. *The University of Maryland at Baltimore is an Affirmative Action/Equal Opportunity/ADA Employer, and has a strong institutional commitment to the principal of diversity in all areas.*

MYRIAD GENETICS, INC.

Positions Available

Myriad Genetics, located adjacent to the University of Utah and in the foothills of the Wasatch Mountains, is a publicly-held genomics company focused on the isolation of significant disease-causing genes. Our scientific achievements include elucidating the role of the p16 tumor suppressor gene in human cancer (Science 264: 436-440), and the identification of the breast and ovarian cancer susceptibility gene BRCA1 (Science 266: 66-71). We currently have active programs in the areas of cancer, coronary heart disease, asthma, obesity, and osteoporosis. We also have major corporate collaborations with Ciba Geigy and Bayer pharmaceutical companies. We currently have the opportunity to expand our research efforts in the following areas:

Project Director: Requires a Ph.D. or equivalent, postdoctoral training, and at least five years of independent research experience. The ideal candidate will possess a strong background in molecular biology or genomics research and proven managerial ability.

Research Associates: Requires an M.S. or B.S. degree and a minimum of two years' molecular biology experience, or a Ph.D. without postdoctoral training. ABI sequencing and/or linkage analysis experience is preferred.

Scientific Applications Programmer: An M.S. degree in scientific or numerical subject and significant experience with C in a UNIX environment are essential.

Database Developer & Administrator: Requires a B.S. degree or equivalent and two years of experience with SYBASE, C, and UNIX.

Experience in genetics and DNA sequence analysis is preferred for both programmer positions. Macintosh experience is desirable.

We offer a competitive compensation package and a highly stimulating, interactive environment. Please send or fax your letter of interest specifying position, a complete resume, and the names of three professional references to: Barbara Berry, Human Resources Dept. SCI, Myriad Genetics, Inc., 390 Wakara Way, Salt Lake City, UT 84108. Fax: 801-584-3640. EOE.

MYRIAD

MOLECULAR BIOLOGISTS

There's no better place to pursue a research career than with CIBA, a company recognized worldwide for innovative drug therapies that fulfill unmet medical needs. As part of an ongoing commitment to research excellence, we have several opportunities for molecular biologists with proven research experience.

Join a world class research effort on the forefront of discovery.

Working with a multidisciplinary team of scientists involved in drug discovery research, you will use your knowledge of molecular biology to identify novel cellular targets for drug discovery in cardiovascular disease. Qualified candidates should have experience in the molecular techniques used to study signal transduction pathways and gene expression. A bachelor's degree and lab experience is required; a master's degree is preferred.

We offer a competitive salary, excellent benefits and an environment designed for professional development. For confidential consideration, please send your resume with salary requirements to: **Staffing Center, Ciba Pharmaceuticals Division, Department JN-398, 556 Morris Avenue, Summit, NJ 07901.** We are an equal opportunity employer M/F/D/V.

Preparing for the future

ciba

Microcide Pharmaceuticals Inc. has several positions for highly motivated and qualified individuals in our **Discovery Biology Department.**

Research Associates for positions in molecular microbiology. Qualified applicants will have a BA/BS or MA/MS and experience in a research laboratory using techniques of bacterial genetics and molecular biology (including recombinant DNA techniques and PCR amplification). Macintosh literacy is desired.

High Throughput Screening. Qualified applicants will have at least 4 years experience in high-throughput screening, strong creative skills, supervisory skills and a desire to undertake a challenging high throughput screening/ database management position. Experience in anti-infective screening, the evaluation of natural product and synthetic compound leads, and screen design are preferred. Knowledge of antibacterial agents, bacterial genetics and physiology are desirable.

Microcide is an innovative San Francisco Bay Area pharmaceutical company focused on the discovery and development of novel antimicrobial agents for the treatment and prevention of human infections of bacterial origin. We have exciting opportunities for persons with initiative, flexibility and energy. We offer a competitive salary, excellent benefits and equity participation. Good communication skills, a teamwork orientation, and commitment to excellence are necessary for all positions.

Please send or FAX your resume to: **Employment Officer - Discovery Biology Department, Microcide Pharmaceuticals, 850 Maude Avenue, Mountain View, CA 94043. FAX (415) 428- 3534.** *An equal opportunity employer.*

Research Scientist

Neurophysiology

Lockheed Martin Engineering & Science Services is seeking candidates for a research position on our life sciences support contract at the NASA/Ames Research Center (ARC) in Mountain View, California. The research program at ARC, led by Dr. David Tomko, utilizes unique ground research facilities (including acceleration sleds and centrifuges) and spaceflight experiments, to study the effects of microgravity and acceleration on the vestibular system and vestibulo-ocular reflexes.

We are interested in individuals who will complement the existing research team. Acceptable candidates will have a Ph.D. in life sciences or bioengineering and 0-3 years relevant research experience. Background in computerized data collection and analysis is preferred. Training in rodent and primate surgery is valuable.

Applicants should have strong written and verbal communication skills in English and the ability to work successfully as part of an integrated research team. A publication record should reflect the ability to make independent contributions to a research program.

Lockheed Martin offers competitive salaries and comprehensive benefits. For consideration, please forward your resume or curriculum vitae to: Ms Blythe Asagi, Human Resources, Lockheed Martin Engineering & Science Services, P.O. Box 168, Moffett Field, CA 94035-0168. Lockheed Martin is an equal opportunity, affirmative action employer.

LOCKHEED MARTIN

Engineering & Science Services

THE STATE UNIVERSITY OF NEW JERSEY

RUTGERS

GRADUATE RESEARCH TRAINING in CELLULAR/MOLECULAR BIODYNAMICS

Applications are accepted immediately for multidisciplinary Ph.D. program in CELLULAR and MOLECULAR BIODYNAMICS, funded by the NSF and consisting of research faculty from the Departments of Chemistry, Biological Sciences, Physics, and Behavioral and Neural Sciences. The students receive 5 years of full support ($14,000 + tuition). There is a strong emphasis on training in research techniques and strategies in BIOPHYSICS with special courses in: Biological Spectroscopy, Biological Optics and Cellular Biophysics. General areas of research training in laboratories well equipped with state-of-the-art instrumentation include: Biochemistry, Biophysics, Biomolecular Spectroscopy, Computational Modeling, Immunology, Laser Optics, Light and Electron Microscopy, Molecular Biology, Neuropharmacology, Neurochemistry and Neurophysiology. Qualified UNDERGRADUATE STUDENTS are also invited to apply for summer research internships ($2500 stipend). Applicants must be citizens or permanent residents of the US.

For further information and application forms, contact: **Dr. Frank Jordan, Director, Program in Cellular and Molecular Biodynamics, Department of Chemistry, Rutgers University, Newark, NJ 07102.** FAX 201-648-1264; e-mail:frjordan@andromeda.rutgers.edu.

Rutgers is an EE/AA employer and our Program specifically invites and encourages applications from women, minorities and the physically challenged.

FELLOWSHIP PROGRAMS

UNITED STATES - ISRAEL

BARD, The United States-Israel Agricuitural R&D Fund, announces two Fellowship Programs promoting cooperative agricultural research.

The POSTDOCTORAL FELLOWSHIP PROGRAMS provide support for candidates who are citizens of either the United States or Israel and who have fulfilled the requirements for the Ph.D. degree within the last three years. American candidates must carry out their fellowship research in Israel and Israeli candidates must carry out their fellowship research in the United States. The initial award is for one year and, under certain conditions, a one year extension will be considered. The award for the first year is $27,000 for living and travel expenses and a one time, $2,000 allowance for children (one or more). This is paid directly to the fellow. An allowance not exceeding $3,000 may also be paid to the host institution for operating expenses incurred in the research.

The RESEARCH FELLOWSHIP PROGRAM is intended to support citizens of the United States who are established research scientists in American non-profit research institutions, universities or federal and state agencies during a period of 4 to 12 months in Israel. The grant is $3,000 per month and will be paid in coordination with the base institution of the fellow. One round trip air ticket will be provided.

Applications for either program must reach BARD's offices by **January 15.**

For information on application procedures please contact BARD's offices at:

BARD
USDA-ARS-OIRP
Room 102, Bldg. 005, BARC-West
Beltsville, MD 20705
U.S.A.

Tel: 301-504-5605
Fax: 301-504-5298

BARD
The Volcani Center
P.O.B. 6
Bet Dagan
Israel 50250

Tel: 972-3-9655133
Fax: 972-3-9662506

Global Career Opportunities

THE HONG KONG UNIVERSITY OF SCIENCE AND TECHNOLOGY

SCHOOL OF SCIENCE

Head of Department of Biology

The Hong Kong University of Science and Technology (HKUST) was opened in October 1991 as a technological university with special emphasis on research, graduate education, and close collaboration with industry and business. Enrollment is expected to grow to 7,000 (FTE) by 1997/98. The School of Science which comprises five departments : Biochemistry, Biology, Chemistry, Mathematics, and Physics enrolls about 1/4 of the University's undergraduate and graduate students.

The Department of Biology has comprehensive teaching and research programmes which cover all levels of biological organization, with emphasis on the molecular and cellular levels. The following academic degrees are offered: Bachelor of Science, Master of Science, Master of Philosophy and Doctor of Philosophy. Currently, there are 23 faculty members specializing in 1) molecular, cellular and developmental biology, 2) environmental and marine biology, 3) tumor biology, 4) neurobiology, and 5) plant biology. By the academic year 1997/98, the faculty size will grow to 26.

Qualifications and Experience

Applications/nominations are invited from well-qualified and accomplished biologists for the position of Head of the Department of Biology. In addition to extensive teaching and research experience, the successful candidate must have demonstrable leadership qualities necessary to lead and manage the Department.

Salary and Conditions of Service

Salary will be highly competitive. Generous fringe benefits including medical and dental benefits, annual leave, housing and children's education allowances are provided; air passages are also provided where applicable. Initial appointment will be on a three-year contract, a gratuity of an amount equal to 25% of the total basic salary drawn will be payable upon successful completion of contract.

Application/Nomination Procedures

Applications/nominations together with a detailed curriculum vitae and the names and addresses/fax numbers of three to five referees should be sent to the Director of Personnel, The Hong Kong University of Science and Technology, Clearwater Bay, Kowloon, Hong Kong (Fax No.:(852) 2358-0700) **before 31 January 1996**. Please quote Ref. BIOL.HD on your application/nomination. The search will continue until a suitable appointment is made.

George Mason University

Director, Molecular Bioscience and Technology Institute (MBTI)

In 1997, the university will open a new facility for research and education in molecular bioscience and technology. The Molecular Bioscience and Technology Institute is a newly formed unit that will be located at the Prince William Campus of George Mason University, adjacent to the new laboratory of the American Type Culture Collection (ATCC). GMU and ATCC will have a joint interest in the MBTI as it is the principal vehicle for their cooperative academic enterprise. The MBTI and ATCC will serve as the nucleus for a university and commercial biotechnology center in Northern Virginia.

The university is seeking an Institute Director with an outstanding record in research and research management and a capability for developing innovative academic programs at the M.S. and Ph.D. levels to be integrated with the research effort. The candidate must have demonstrated significant ability as an institution developer. Toward this end, there will be a substantial number of new faculty positions in the Institute in areas of scientific expertise chosen in consultation with the Director. The Director will also relate closely to ATCC management and staff in the GMU-ATCC cooperative academic enterprise.

The Director should qualify for a full professorship and will have a twelve-month administrative appointment at a competitive senior salary. The position is available immediately. Nominations and applications will be accepted until the position is filled and should be sent to **Harold J. Morowitz, Chair, Search Committee, Krasnow Institute for Advanced Study at George Mason University, Mail Stop 2A1 , George Mason University, Fairfax, VA 22030.** *Review of applications will begin on February 1, 1996.*

AA/ EOE Employer

Global Career Opportunities

UNIVERSITY OF DUBLIN

TRINITY COLLEGE

CHAIR OF GEOLOGY AND MINERALOGY (1844)

Applications are invited for appointment to the Chair of Geology and Mineralogy which has become vacant on the retirement of Professor C H Holland.

The successful applicant will have a distinguished reputation and an established record of research in any of the constituent disciplines of the subject and will be expected to provide academic leadership through a strong commitment to teaching and research.

The appointment will be effective from 1 October 1996 or as soon as possible thereafter.

Information about the Department of Geology and further particulars of the appointment, including details of salary and other benefits, may be obtained from:

Michael Gleeson
Secretary to the College
West Theatre
Trinity College
Dublin 2
Telephone: +353 1 608 1123/2197
Fax: +353 1 671 0037
e-mail: secretary@tcd.ie

The preferred closing date for receipt of applications is **31 January 1996.**

Trinity College is an equal opportunities employer.

Associate Dean for Research and Assistant Director of the Minnesota Agricultural Experiment Station

College of Agricultural, Food, and Environmental Sciences University of Minnesota, Twin Cities

The College of Agricultural, Food, and Environmental Sciences and the Minnesota Agricultural Experiment Station (MAES) seek an Associate Dean for Research and an Assistant Director of MAES. The successful applicant will report directly to the Dean and will assist the Dean in managing and coordinating research activities. He or she will also have primary responsibility and authority for administering the five Branch Stations. Salary is open and commensurate with experience.

Qualifications

The successful applicant for this position must have an earned doctorate in an agricultural, biological, environmental, or food-related field and five years' professional, post-doctorate research experience. Desired qualifications include research experience beyond the five-year minimum; a record of productive research, education, and scholarly activity; research or administrative experience in an academic institution; administrative experience involving familiarity with budgets and finance; demonstrated leadership skills; demonstrated familiarity with state and federal research and experiment station systems and with agricultural production; familiarity with interdisciplinary research; demonstrated excellence in written and verbal communication; and demonstrated commitment to affirmative action, cultural diversity, and equal opportunity for women and minorities.

Send all applications, nominations, and requests for complete position descriptions no later than **January 8, 1996** to:

Billie Wahlstrom, Ph.D.
Chair, Search Committee
201 Haecker Hall
University of Minnesota
St. Paul, MN 55108-1030
Telephone: (612) 624-3445
Fax: (612) 624-3617

U of M is an EEO educator and employer.

Global Career Opportunities

香 港 大 學
THE UNIVERSITY OF HONG KONG

Lectureship/Assistant Lectureship in Physiology

Applications are invited for a Lectureship/Assistant Lectureship (non-clinical) in the Department of Physiology (RF-95/96-53), tenable from 1 April 1996 or as soon as possible thereafter. Appointment will be made on a fixed-term basis of three years, with a possibility of renewal. Applicants should possess a PhD, MBBS, MD or equivalent qualification, with demonstrated ability to establish an independent and extramurally fundable research programme which complements ongoing programmes, and to teach Physiology to undergraduate and postgraduate students. Two to three years of post-doctoral experience in Physiology or related research areas with excellent organizational, supervisory and communication skills are desirable. Applicants should indicate clearly which level they wish to be considered for.

Annual salaries [non-superannuable but attracting 15% (taxable) terminal gratuity] are on the following scales, with starting salary depending on qualifications and experience: Lecturer HK$454,200 - HK$758,700 (11 points: approx. US$58,987 - US$98,532); Assistant Lecturer HK$330,780 - HK$423,360 (4 points: approx. US$42,958 - US$54,981). US$ equivalents as at 9 November 1995.

At current rates, salaries tax will not exceed 15% of gross income. Children's education allowances, leave, and medical benefits are provided; housing or tenancy allowances are also provided in most cases at a charge of a percentage of salary, currently 7½%.

Further particulars and application forms may be obtained from the Appointments Unit, Registry, The University of Hong Kong, Hong Kong (fax: [852] 2559 2058; E-mail:APPTUNIT@REG.HKU.HK). Particulars are also available on the University's listserv accessed by E-mail as "listserv@hkuvm1.hku.hk" (specify "get apptment filelist" for list of vacant posts). Closes **2 January 1996.**

Global Career Opportunities

NFR Naturvetenskapliga forskningsrådet
Swedish Natural Science Research Council
Box 7142 103 87 Stockholm Tel 08-454 42 00 Fax 08-454 42 50

invites applications for

RESEARCH POSITIONS in

Analogue Tectonic Modelling
Astrophysics
Asymmetric Synthesis
Atomic and Molecular Physics
Biomolecular Structure Transitions and Dynamics
Couplings between the Biogeochemical Cycle and Global Climate
Global Scale Dynamics of the Biogeosphere, particularly Soil-Water Interaction
Non-Perturbative Quantum Theories
Nucleotide and Nucleoside Chemistry
Reaction Dynamics

The Council's intention in creating these posts is to contribute to the recruitment of researchers and to bring new life into research. The programme is, therefore, aimed primarily at early to mid career researchers. These positions will be at an appropriate university department within Sweden to be chosen in consultation between the candidate, NFR and the university. Tenure will be for three years as from July 1, 1996 and may be prolonged for another three years. The salary range will correspond to that of an associate professor (SEK 300 000-350 000 per year, ie USD 44 000-52 000 per year). Applications should reach the Swedish Natural Science Research Council, Box 7142, S-103 87 Stockholm, Sweden, by February 15, 1996. A curriculum vitae including a list of publications, a short research plan and a maximum of 10 reprints of scientific papers should be appended in four copies to the application which should also be in quadruplicate. Further information can be obtained from Ms. Natalie Lunin at the Secretariat of NFR, phone no. +46 8 454 42 32, fax no. +46 8 454 42 50.

Global Career Opportunities

UNIVERSITY OF DUBLIN
TRINITY COLLEGE

UNIVERSITY CHAIR OF BOTANY (1711)

Applications are invited for appointment to the Chair of Botany (1711). The Department has established strength in research in the fields of ecology, ecophysiology, palaeoecology, taxonomy and mycology. This includes special interests in carbon fixation and partitioning, forest ecology, soil plant relationships, aerobiology, bioindicators, and the taxonomy of S E Asian floras. Collaboration is active with groups throughout Ireland and the rest of Europe, North and South America, East Africa, S E Asia and Australia. The resources of the Department include a major herbarium and a botanic garden.

The Department of Botany's main teaching contribution is to its 4 year Honors course leading to a B A (Moderatorship) in Botany. Contributions are also made to the Moderatorship courses in Environmental Sciences and Earth Sciences.

The successful applicant will have a distinguished reputation in research and will be expected to provide strong leadership in both teaching and research. The person appointed will serve as Head of Department in accordance with College regulations concerning headship.

The appointment will be effective from 1 October 1996 or as soon as possible thereafter.

Information about the Department of Botany and further particulars of the appointment, including details of salary and other benefits, may be obtained from:

Michael Gleeson, Secretary to the College
West Theatre, Trinity College. Dublin 2
Telephone: +353 1 608 1123/2197/Fax: +353 1 671 0037
e-mail: secretary@tcd.ie

The preferred closing date for receipt of applications is: **31 January 1996.**

Trinity College is an equal opportunities employer.

Royal Netherlands Academy of Arts and Sciences calls for nominations for the Heineken Prizes 1996

The Royal Netherlands Academy of Arts and Sciences (KNAW) selects the recipient of the

- Dr H.P. Heineken Prize for Biochemistry and Biophysics 1996
- Dr A.H. Heineken Prize for Medicine 1996
- Dr A.H. Heineken Prize for Environmental Sciences 1996

These biennial prizes, offered by the Heineken Foundation and the 'Alfred Heineken Fondsen' Foundation, rewards outstanding scientific achievement in the field of biochemistry and biophysics, medicine and environmental research.

Along with a permanent token each Prize consists of Dfl. 250,000.-- (about US $ 160,000.--) to be spent at will.

Nominations are restricted to individual, active scientists whose contributions to biochemistry and biophysics, medicine or environmental research should be outstanding and a source of inspiration to others.

Nominations must include a description of the research work and publications on which the nomination is based, a curriculum vitae, bibliography, and one or two key publications. The deadline for nominations is January 1, 1996.

Nomination forms and general information are available from the

Secretariat of the Heineken Prizes,
Royal Netherlands Academy of Arts and Sciences
P.O.Box 19121, 1000 GC Amsterdam, The Netherlands

Telephone: 31 (0) 20 55 10 769, telefax: 31 (0) 20 6 20 49 41

Royal Netherlands Academy of Arts and Sciences

UNIVERSITY OF NATAL

KWAZULU-NATAL PROVINCIAL ADMINISTRATION SOUTH AFRICA

FACULTY OF MEDICINE

Applications are invited from suitably qualified persons for appointment to the following posts which are on the Joint Medical Staff Establishment carrying University conditions of service.

Remuneration will be in accordance with that specified by the Public Service and will be made known upon request.

(Chief Specialist) Professor and Head of the Department

DEPARTMENT OF HUMAN ANATOMY

ADV: MS 15/95 **Closing date: 28.02.1996**

Applicants must have a higher qualification in Anatomy or some aspect of clinical medicine and be registrable with the South African Medical and Dental Council as a Specialist. Experience in undergraduate and postgraduate medical education together with a strong academic background and a proven research record are University prerequisites. Candidates will be judged on their academic and service performance, research background, educational experience and administrative ability.

DEPARTMENT OF VIROLOGY

ADV: MS 18/95 **Closing date: 28.02.1996**

Applicants must have a higher qualification in Virology and be registrable with the South African Medical and Dental Council as a Specialist Virologist or have appropriate experience as a medical scientist. Experience in undergraduate and postgraduate medical education together with a strong academic background and a proven research record are University prerequisites. Candidates will be judged on their academic and service performance, research background, educational experience and administrative ability.

These are full time posts, however, limited private remunerative work may be considered upon application.

Access to teaching facilities, communities and laboratories provide opportunities for research across a wide clinical spectrum.

Application forms are available from the Personnel Section, Faculty of Medicine, P O Box 17039, Congella 4013, South Africa, Telephone +27-31-260-4419. Fax: +27-31-260-4410.

A major French pharmaceutical company is seeking for its research centre located in the west area of Paris a young scientist for a

POST-DOCTORAL POSITION

Cardiovascular Research

Qualifications include a PhD in biochemistry, cell or molecular biology and practical experience of classical techniques used in these fields. The position is an excellent opportunity to share and to gain valuable experience in a stimulating environment.

The successful candidate will join a pharmacology department active in cardiovascular drug discovery and will be involved in the study of the pathophysiology of the vascular wall (endothelial dysfunction, proliferation of vascular smooth muscle cells).

The position is vacant for 18 months.

Applicants are invited to send a curriculum vitae with a handwritten letter and a photo to Media System, 6 impasse des Deux Cousins 75849, Paris Cedex 17 France, quoting reference 19396.

MEDIA SYSTEM

dkfz

Deutsches Krebsforschungszentrum
Stiftung des öffentlichen Rechts

Universität Heidelberg

The German Cancer Research Center (DKFZ) and the University of Heidelberg are establishing a clinical cooperation unit of Dermato-Oncology at the Department of Dermatology of the Klinikum-Mannheim, in order to promote cooperation between basic research , clinical research and patient care. The clinical cooperation unit will be a division of the DKFZ and located initially for a five year period within the premises of the Department of Dermatology at the Klinikum Mannheim. Tenure will depend upon the success of the cooperation and the scientific results.

The head of the clinical cooperation unit will be offered a

Professorship (C3) for Dermato-Oncology

at the Faculty of Medicine at Mannheim, University of Heidelberg.

Preference will be given to applicants with a focus on basic research in molecular and cell biology. Innovative and substantial contributions to the development of basic research into clinical applications, including methods of gene technology, are expected.

Medicinal licence ("Approbation") is required, board certification in Dermatology and "Habilitation" or equivalent scientific work are expected.

Applications with a curriculum vitae and a complete list of publications should be sent to:

Deutsches Krebsforschungszentrum, Wissenschaftlicher Stiftungsvorstand Im Neuenheimer Feld 280, D-69120 Heidelberg by 31 January 1996.

The Chinese Academy of Sciences
with the support of
the Max-Planck-Gesellschaft
intends to establish the second
Independent Research Group
at the Shanghai Institute of Cell Biology
for a
Promising Chinese Scientist
Applications are invited for a position as a
Research Group Leader

from scientists from China or abroad who have achieved a degree of international recognition in their field, preferably in
Developmental Biology
Cell Biology
Molecular Biology or
Neurobiology

The Research Group Leader will be expected to conduct fully independent, original and dynamic research programs.

The position commands full scientific and economic independence and is limited to a maximum of five years; it is paid according to Chinese regulations. In addition, special social benefits, a supplement in foreign currency and travel grants are offered. The group will have modern laboratory facilities and funding for scientific and technical co-workers. The operating expenses include funds in foreign currency.

Applications, including a tabular curriculum vitae, a list of publications with reprints of three selected papers, a description of major scientific achievements and a summary of future research plans, should be submitted in English by January 15th 1996, to:

Professor Uli Schwarz (director)
Max-Planck-Institut für Entwicklungsbiologie
Postfach 2109, 72011 Tübingen, Germany
Fax No. 0049-7071-601447
Applicants from China are strongly encouraged to submit their applications.
A group of finalists will be invited to present their projects at a symposium in Shanghai in the first half of April 1996. Travel and accommodation expenses will be covered.

KAROLINSKA INSTITUTET
Department of Bioscience at Novum
Center for Structural Biochemistry

Research assistant/ Postdoctoral fellow
NMR spectroscopy in biology

The position is available within the Unit for NMR Spectroscopy at the Center for Structural Biochemistry (CSB), at the Department of Bioscience. The CSB is situated at the South Campus of the Karolinska Institutet encompassing the NOVUM Research Park in Huddinge, about 20 km south of the center of Stockholm. The CSB has a staff of about 30 researchers and includes units for X-ray crystallography, molecular modelling, electron microscopy and NMR spectroscopy. The NOVUM Research Park provides an excellent environment with over 400 researchers within various biomedical disciplines.

The research within the Unit for NMR Spectroscopy concerns structural and biophysical studies of biomolecules. Present projects include studies of nuclear receptors, PCB-binding proteins, ribosomal proteins and DNA-binding proteins from archaebacteria. The NMR unit is equipped with a Varian Unity 500 MHz NMR spectrometer with all necessary hardware and extensive computing resources.

The applicant should have a background in the application of NMR spectroscopy for studies of biomolecular structure and dynamics. A second condition is that applicants should have experience in protein expression and/or protein biochemistry. The position is available for four years with an initial limitation of one or two years.

For further information contact Dr. Torleif Härd (email: th@csb.ki.se) (questions regarding qualifications etc.) or Ingwar Lennerfors (administrative questions) +46-8 608 92 23.

Applications should be sent to Karolinska Institutet, Department of Bioscience at Novum, S-141 57 HUDDINGE, SWEDEN together with CV, relevant publications, a short description of research interests and two recommendation letters. Please quote reference no. 53021/8 and note that we want your application in 1 original and 1 copy.

Closing date: December 22, 1995

POSITIONS OPEN

SCIENCE WRITER—The Office of Public Affairs of a scientific society representing 25,000 members seeks a well-organized **SCIENCE WRITER**. Responsibilities include preparing news releases, a news column on issues that affect science, and newsletter articles explaining the importance of basic neuroscience research. Experience required: at least five years of science reporting and writing, familiarity with neuroscience, and proven ability to make complicated concepts understandable to the public. Knowledge of science funding and congressional science politics is helpful. Must be able to handle multiple projects and respond quickly to changing demands. Excellent benefits, including health and pension plans. Salary is around $40,000. Mail cover letter, résumé, and three clips of science articles before January 1, 1996 to: **Science Writer, Society for Neuroscience, 11 Dupont Circle NW, Suite 500, Washington, DC 20036**. No telephone calls please.

CAREER CHANGE OPPORTUNITY

This unique program offers the candidate with an earned doctorate in the sciences the opportunity to obtain the Doctor of Optometry (O.D.) degree in two calendar years. Employment opportunities exist in private practice, industry, education, and research. Contact: **Dr. D. Chauncey, Program Director, Accelerated Program, The New England College of Optometry, Room S, 424 Beacon Street, Boston, MA 02115.**

Medical University of South Carolina (MUSC)

Offers programs leading to Ph.D. and M.D./Ph.D. degrees in each of the basic biomedical sciences (biochemistry, microbiology/immunology, pathology, pharmacology, physiology), biometry/epidemiology, pharmaceutical sciences, and molecular and cellular biology. Competitive stipends of $15,000 available. Contact: **College of Graduate Studies, MUSC, 171 Ashley Avenue, Charleston, SC 29425-2501.**

TECH SEARCH INTERNATIONAL

Positions available nationwide. Permanent and temporary. R&D, validation, production, QA/QC and executive levels. All disciplines, B.S., M.S., Ph.D., M.B.A. Mail résumé to: **Tessie R. Cuy, Technical Director, P. O. Box 2302, Dublin, CA 94568. Telephone: 510-829-1327; FAX: 510-829-1727; Email: trcuy@aol.com.**

COURSES AND TRAINING

SUMMER INSTITUTE IN STATISTICAL GENETICS
June 6–18, 1996
North Carolina State University

The Institute will consist of a series of short courses aimed at geneticists wishing to acquire statistical skills as well as statisticians wishing to become acquainted with genetic applications. The courses will cover statistical aspects of population and quantitative genetics, sequence analysis, QTL mapping, and forensic calculations. Contact: **Dr. B. S. Weir, Department of Statistics, North Carolina State University, Raleigh, NC 27695-8203**, visit WWW at **http://www2.ncsu.edu/ncsu/CIL/stat_genetics.**

BIOLOGY OF DISEASE VECTORS COURSE
COLORADO STATE UNIVERSITY
FORT COLLINS, COLORADO, USA
16 June to 28 June, 1996

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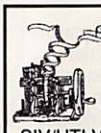

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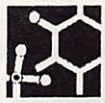

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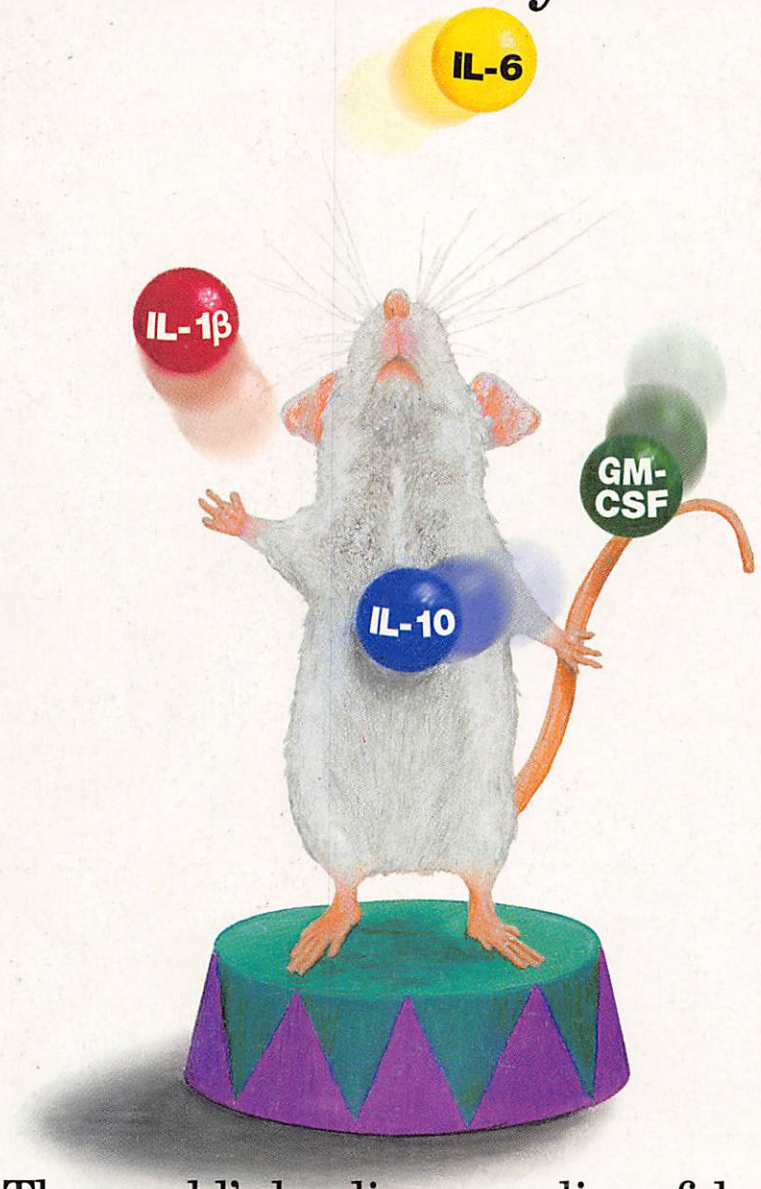

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